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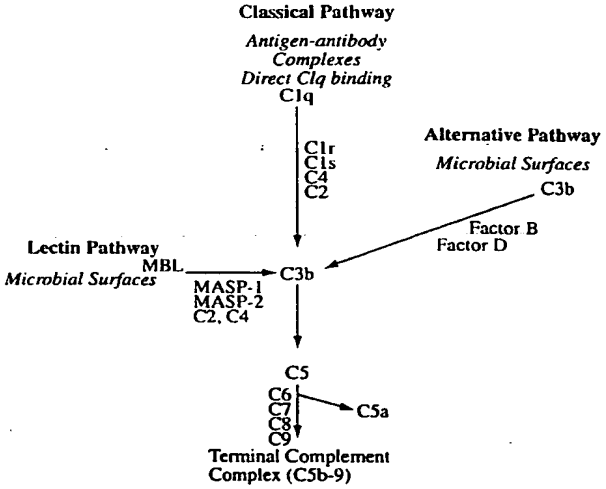

(57) Abstract: The invention relates to methods and products for regulating lectin complement pathway associated complement activation. The methods include both *in vitro* and *in vivo* methods for inhibiting lectin complement pathway associated complement activation. The methods are accomplished by contacting a mammalian cell having surface exposed MBL ligand with an effective amount of a mannan binding lectin (MBL) receptor antagonist to inhibit lectin complement pathway associated complement activation. The mannan binding lectin receptor antagonist may be administered to a subject to prevent cellular injury mediated by lectin complement pathway associated complement activation. The products of the invention include compositions of a mannan binding lectin receptor antagonist. The mannan binding lectin receptor antagonist is an isolated mannan binding lectin that selectively binds to a human mannan binding lectin epitope and that inhibits lectin complement pathway associated complement activation.

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INHIBITORS OF THE LECTIN COMPLEMENT PATHWAY (LCP) AND THEIR USE

Field of the Invention

The present invention relates to methods and products for regulating lectin complement pathway (LCP) associated complement activation. In particular, the invention relates to methods for inhibiting LCP associated complement activation by contacting a mammalian cell having a mannose binding lectin (MBL) ligand (alternatively referred to herein as an "MBL receptor") with an antagonist of MBL. The invention also relates to novel complement inhibitors which are MBL receptor antagonists, such as plant lectins and functional equivalents thereof.

Background Of The Invention

The immune system functions to defend the body against pathogenic bacteria, viruses and parasites. Immunity against foreign pathogens usually involves the complement system. The complement system is a cascade of 18 sequentially activated serum proteins which function to recruit and activate other cells of the immune system, effect cytolysis of target cells and induce opsonization of foreign pathogens. Complement can be activated by the presence of either antibody/antigen complexes, as in the classical complement pathway, or microbial surfaces, as in the alternative complement pathway. Complement activation can also occur via the lectin complement pathway (LCP). Lectins are carbohydrate-binding proteins that recognize oligosaccharide structures present on cell surfaces, the extracellular matrix, and secreted glycoproteins. As shown in Figure 1, these distinct activation pathways ultimately converge at the common enzymatic step of serum protein C3 cleavage to C3b and C3a. This in turn initiates the terminal steps of complement function including the cleavage of C5 to C5b and C5a and subsequent deposition of C5b-C9 onto the target cell membrane.

The LCP is an antibody-independent cascade that is initiated by binding of mannan- (or mannose) binding lectin (MBL) to cell surface carbohydrates on bacteria, yeasts, parasitic protozoa, and viruses (Turner MW, "Mannose-binding lectin: The pluripotent molecule of the innate immune system", *Immunol.Today,* 1996;17:532-540). MBL (~600 kDa) is a member of the collectin protein family and is structurally related to the classical complement C1 subcomponent, C1q. Associated with MBL are two serine proteases, Mannose binding lectin

associated serine protease, MASP-1 and MASP-2, which show striking homology to the two C1q-associated serine proteases of the classical complement pathway, C1r and C1s (Thiel S, et al., "A second serine protease associated with mannan-binding lectin that activates complement", *Nature* 1997;386:506-510). The selectivity of MBL sugar binding is: N-acetyl-D-glucosamine (GlcNAc) > mannose > N-acetylmannosamine and fucose > maltose > glucose >> galactose and N-acetylgalactosamine (Thiel S, et al., "A second serine protease associated with mannan-binding lectin that activates complement", *Nature* 1997;386:506-510; Turner MW, "Mannose-binding lectin: The pluripotent molecule of the innate immune system", *Immunol.Today,* 1996;17:532-540). Binding of the MBL/MASP complex to cell surface carbohydrates activates the LCP, which in turn activates the classical complement pathway independently of C1q, C1r, C1s or antibodies (Fig. 1). Most if not all the carbohydrate moieties to which MBL binds are not normally expressed by unperturbed human tissue.

Summary Of The Invention

The present invention relates to methods and products for regulating lectin complement pathway (LCP) associated complement activation. Prior to the instant invention, it was known that LCP associated complement activation was a mechanism used by the body to recognize and destroy an invading microorganism. LCP activation normally occurs through the binding of mannan-binding lectin (MBL) and its two associated serine proteases, MASP-1 and MASP-2, to carbohydrates on the surface of microorganisms. Once MBL and MASP-1 and MASP-2 are localized to the surface of the microorganism, complement begins to assemble, ultimately killing the microorganism. These prior art teachings demonstrate that MBL is an important cellular component in the process of the eradication of infectious microorganisms. In fact, MBL deficiencies can result in medical disorders. A disease known as MBL deficiency, in which children that are deficient in MBL, renders the children prone to the development of infectious diseases.

Recent findings from our laboratory have demonstrated that the mechanism of complement activation following oxidative stress of human endothelial cells is initiated by mannose-binding lectin (MBL) deposition to an endothelial cell ligand. Thus, specific inhibition of MBL or its counter-ligand on endothelial cells appeared to us to be an attractive therapeutic strategy for treating conditions characterized by activation of the MBL

complement activation pathway. In view of our findings, we have investigated the molecular mechanism of complement activation during oxidative stress of endothelial cells using novel therapeutics directed against the endothelial MBL ligand. Thus, the experiments described herein were undertaken with the general aim of characterizing the molecular mechanisms governing complement activation following oxidative stress of endothelial cells and to develop novel, small molecular weight complement inhibitors directed towards inhibition of MBL deposition on endothelial cells. To this end, we have identified several legume-derived lectins [e.g., *Ulex europaeus* (UEA)-II and *Laburnum alpinum* (LAA)-I] that bind to the MBL ligand and inhibit complement activation.

Additionally, we have designed several peptide-based MBL receptor antagonists that display molecular mimicry to N-acetylglucosamine (i.e., a specific inhibitor of MBL), and/or have similar functions. Some of these peptides were designed by using the known amino acid sequence of cytokeratin (K) 14 and 17, which belong to the larger cytokeratin family members of which we have identified as likely MBL ligands on stressed endothelial cells. Although not wishing to be bound to any particular theory or mechanism, we believe that Cytokeratin (K) expression is responsible for complement activation following oxidative stress of human endothelial cells. Accordingly, we believe that inhibition of K with naturally occurring legume lectins, functional equivalents of these lectins, or other specific anti-cytokeratin inhibitors, e.g. antibodies, antibody fragments, binding peptides, is useful for inhibiting MBL deposition and the resulting complement activation following oxidative stress in vitro and in vivo.

Our understanding of the role of cytoskeletal filaments in cellular function has significantly advanced in recent years. In addition to providing structural support, it is now clear that intermediate filaments play a key role in a variety of cellular functions, including cell-cell and cell-extracellular matrix interactions, cell motility, receptor-ligand interactions, and receptor internalization (Pavalko and Otey, Proc. Soc. Exxp. Biol. Med, 205, 282-293, 1994 and Fuches and Cleveland, Cell Motif Cytoskelaton, 17, 291-300, 1990). Although various intermediate filaments exists in human endothelial cells, their non-structural roles have not been fully elucidated. Intermediate filaments were previously reported to activate the "classical" complement pathway in an antibody independent fashion (Linder et al., Nature, 278, 176-177, 1979; Linder et al., Clin. Imm. and Immunopath., 40, 265-275, 1986). Recently, the intermediate filament cytokeratin 1 (CK1) was cloned from a human

endothelial cell library and identified as a kininogen binding protein (Joseph et al., Clin. Immunol. 92, 246-255, 1999; Joseph et al., Immunopharmacology, 43, 203-210, 1999; Shariat-Madar et al., J. Biol. Chem. 274, 7137-7145, 1999; Hasan et al., Proc. Natl. Acad. Sci USA 95:3615-3620, 1998), suggesting that endothelial cytokeratins may function as extracellular binding proteins.

Thus, in one aspect, the present invention is based upon the surprising discovery that certain plant lectins (e.g., UEA-II, LAA-1, Cytisus Sessilifolius anti-H(O) Lectin 1 (CSA-1), and functional equivalents thereof) have a similar binding profile as MBL with respect to recognizing specific carbohydrates or peptides on the surface of mammalian endothelial cells and competitively inhibit MBL deposition and subsequent complement activation of HUVECs following oxidative stress and thus are MBL receptor antagonists. Surprisingly, it has also been discovered that certain plant lectins function as receptor antagonists of MBL, thereby inhibiting MBL deposition on the surface of mammalian cells and inhibiting the development of diseased or damaged tissue. In another aspect, the invention is based on the discovery that antibodies, antibody fragments and other keratin binding molecules are also MBL receptor antagonists.

In another aspect, the invention is a method for inhibiting LCP-associated complement activation. The method includes the step of contacting a mammalian cell having a surface exposed MBL ligand with an effective amount of an MBL receptor antagonist to inhibit cellular MBL deposition and LCP-associated complement activation. In one illustrative embodiment, the method is an *in vitro* screening assay.

In yet another aspect, the invention is a method for inhibiting a cellular injury mediated by LCP-associated complement activation. The method includes the step of administering to a subject in need thereof an effective amount of an MBL receptor antagonist to inhibit LCP-associated complement activation.

In one embodiment of the methods of the invention, the MBL receptor antagonist is an isolated molecule that selectively binds to a mannose binding lectin (MBL) ligand (alternatively referred to herein as an "MBL receptor") on a mammalian cell and inhibits MBL binding thereto. In an illustrative embodiment, the isolated MBL receptor antagonist is a binding molecule such as a peptide mimetic of the carbohydrate recognition domain (CRD) of the plant lectins disclosed herein or their functional variants. In another embodiment, the MBL receptor antagonist is a keratin binding molecule.

It is believed that the cellular injury mediated by LCP-associated complement activation contributes to the development of injured tissue associated with a variety of disorders. In one embodiment, the cellular injury is associated with atherosclerosis. In another embodiment, the cellular injury is associated with arthritis, myocardial infarction, ischemia and reperfusion, transplantation, CPB, stroke, ARDS, SLE, Lupus, or dialysis.

The MBL receptor antagonist may be administered to the subject by any route known in the art. When the cellular injury is associated with the pulmonary system, the MBL receptor antagonist may be administered to the subject by an aerosol route of delivery. When the cellular injury is due to ischemia or reperfusion, the MBL receptor antagonist may be locally administered to the heart or arteries that have been subjected to ischemia or reperfusion conditions.

According to another aspect of the invention, an MBL receptor antagonist is provided. The MBL receptor antagonist is an isolated molecule that selectively binds to a human MBL receptor and inhibits LCP-associated complement activation. Although not wishing to be bound to a particular theory or mechanism, it is believed that the MBL receptor antagonists of the invention competitively inhibit MBL binding to its receptor (also referred to herein as its ligand), thereby inhibiting LCP-associated complement activation.

According to still another aspect of the invention, a method for optimizing a selected MBL receptor antagonist for inhibiting LCP-associated complement activation is provided. The method involves identifying molecular mimics of the naturally-occurring peptides and functional equivalents disclosed herein which bind to a human MBL receptor and inhibit LCP-associated complement activation. Such molecular mimics can be identified, for example, by generating a library of closely related compounds and screening the library for compounds which possess the functional characteristics of the MBL receptor antagonists disclosed herein. (See, Gold, L. et al. (1995) *Ann. Rev. Biochem.* 64:763-797 which describes the selection of compounds from a combinatorial library using SELEX technology).

According to yet another aspect, the invention is a composition of an MBL receptor antagonist, wherein the MBL receptor antagonist is an isolated binding molecule that selectively binds to a human MBL receptor and that inhibits LCP-associated complement activation. In an illustrative embodiment the composition is a pharmaceutical composition including an effective amount of the isolated MBL receptor antagonist for treating an MBL

mediated disorder and a pharmaceutically acceptable carrier. In certain embodiments, the compositions also include one or more drugs for the treatment of an MBL mediated disorder.

A method for screening a subject for susceptibility to treatment with MBL receptor antagonist is provided in another aspect of the invention. The method includes the steps of contacting a mammalian cell from a subject with a labeled isolated MBL receptor antagonist, and detecting the presence of an MBL on the surface of the mammalian cell, wherein the presence of the MBL indicates that the cell is susceptible to LCP-associated complement activation and that the subject is susceptible to treatment with an MBL receptor antagonist. In one embodiment, the mammalian cell is an endothelial cell; in yet other embodiments, the mammalian cell is an epithelial cell.

Each of the limitations of the invention can encompass various embodiments of the invention. It is, therefore, anticipated that each of the limitations of the invention involving any one element or combinations of elements can be included in each aspect of the invention.

Brief Description Of The Drawings

This application may include drawings which illustrate various aspects of the invention; however, the drawings are not required for enablement of the claimed invention.

Figure 1 is a schematic depicting the antigen/antibody-dependent classical complement pathway and the antibody-independent alternative and lectin complement pathways. All three pathways merge at C3 and lead to the formation of the terminal complement complex (C5b-9).

Figure 2 is lectin complement pathway activation on keratin-coated plates. 3F8, GlcNAc, or GLUPEP, but not 1C10 (non-functional anti-MBL mAb) inhibited C3 deposition. $P<0.05$ compared to A. N=3.

Figure 3 is an MBL deposition to BSA-coupled GLUPEP. MBL deposit was increased on GLUPEP coupled to BSA (Vehicle) compared to BSA only. GlcNAc (100 mmol/L), 3F8 (1 mg/ml) or GLUPEP (50 ug/ml) attenuated/inhibited MBL deposition to GLUPEP coupled to BSA. Data are the means of 4 wells from one experiment.

Figure 4 is LAA-I inhibiting C3 deposition on HUVECs following oxidative stress. Similar to observations made with UEA-II, this legume lectin decreased C3 deposition in a dose-dependent manner. N=3.

Figure 5 is a graph depicting UEA-II inhibition of C3 deposition on keratin.

Figure 6 is graph depicting GLUPEP inhibition of MBL binding to GlcNAc-BSA.

Detailed Description Of The Invention

The invention relates to methods and products for regulating and manipulating lectin complement pathway (LCP)-associated complement activation. As discussed above, the invention is based on the finding that LCP-associated complement activation plays a role in complement induced cellular injury of mammalian cells and that MBL receptor antagonists inhibit LCP-associated complement activation. It was discovered according to an aspect of the invention that MBL interacts with carbohydrates or peptides on the surface of mammalian cells *in vitro* and *in vivo* and that certain plant lectins and functional equivalents thereof (disclosed herein) function as MBL receptor antagonists. It was also discovered that keratin binding molecules function as MBL receptor antagonists. Accordingly, the MBL receptor antagonists of the invention inhibit the accumulation of surface associated MBL which leads to the accumulation of complement on the surface of the cell, thereby inhibiting cell injury or death. According to the prior art, LCP-associated complement activation was predominantly associated with infectious microorganisms, suggesting that MBL deposition should be promoted in order to enhance the killing of infectious microorganisms. It was discovered, according to the invention, that in mammals it is preferable to block MBL cellular association and that such blockage could be achieved using the MBL receptor antagonists disclosed herein. The LCP is not necessary for eradication of infectious microorganisms in adult mammals, and it is believed that it contributes to cellular injury associated with several types of disorders, such as atherosclerosis, arthritis, myocardial infarction, ischemia and reperfusion injury, transplantation, CPB, stroke, ARDS, SLE, Lupus, and dialysis.

In one aspect, the invention is a method for inhibiting LCP-associated complement activation. The method includes the steps of contacting a mammalian cell of a subject having surface exposed MBL ligand (alternatively referred to herein as an "MBL receptor") with an effective amount of an MBL receptor antagonist to inhibit LCP-associated complement activation.

A "subject" as used herein includes humans, non-human primates, dogs, cats, horses, sheep, goats, cows, rabbits, pigs and rodents.

A "human MBL ligand" or "human MBL receptor" as used herein is a receptor expressed on a mammalian cell which when contacted with an MBL activates LCP-

associated complement activation. An MBL ligand is any cell surface molecule capable of interacting with MBL. One example of an MBL ligand is a cytokeratin. Cytokeratins, such as cytokeratin 1 (CK1), are intermediate filaments found on the cell surface.

The methods of the invention are useful for inhibiting LCP-associated complement activation on the surface of a mammalian cell having surface exposed MBL ligand (carbohydrate or peptide groups) recognized by MBL. The mammalian cell may be any cell in which the cell surface carbohydrates or peptides interact with MBL. In certain illustrative embodiments, the mammalian cell is an endothelial cell or an epithelial cell having a surface exposed MBL ligand. For instance, vascular endothelial cells have been shown in subjects that have sustained ischemic/reperfusion injury to express an MBL ligand. Mammalian cells having MBL ligands can easily be identified. For instance, an MBL binding assay (e.g., such as those described below) can be used to identify MBL ligands.

The method for inhibiting LCP-associated complement activation may be used for a variety of *in vitro* and *in vivo* purposes. The method may be used, for instance, as an *in vitro* screening assay. The *in vitro* screening assay may be used to identify compounds which function as an MBL receptor antagonist, such as the assay described above, to identify mammalian cells having surface exposed MBL ligands, or to detect susceptibility of a subject to treatment with an MBL receptor antagonist. In order to screen a subject for susceptibility to treatment with an MBL receptor antagonist, a cell is isolated from the subject and the presence of MBL or the ability of MBL to bind to the surface is detected. If MBL is present on the surface of a cell or is able to bind to the surface of a cell, then the cell is susceptible to LCP-associated complement activation. If this is the case, then the subject is susceptible to treatment with an MBL receptor antagonist.

The methods of the invention are also useful *in vivo* when it is desirable to inhibit MBL deposition on a mammalian cell surface. For instance, the methods of the invention are useful for treating an MBL mediated disorder. The MBL receptor antagonists can be used alone as a primary therapy or in combination with other therapeutics as an adjuvant therapy to enhance the therapeutic benefits of other medical treatments.

The mammalian cell is contacted with an MBL receptor antagonist. The step of "contacting" as used herein refers to the addition of the MBL receptor antagonist to a medium containing a mammalian cell. The medium may be an *in vitro* tissue culture or a biological specimen, an *ex vivo* sample, or *in vivo*. The step of contacting refers to the

addition of the MBL receptor antagonist in such a manner that it will prevent LCP-associated complement activation associated with the mammalian cell.

An "MBL mediated disorder" as used herein is a disorder which involves cellular injury caused by LCP-associated complement activation. MBL disorders include, for instance, atherosclerosis, arthritis, myocardial infarction, ischemia and reperfusion injury, transplantation, CPB, stroke, ARDS, (Systemic lupus erythematosus) SLE, Lupus, and dialysis. Each of these disorders is well-known in the art and is described, for instance, in *Harrison's Principles of Internal Medicine* (McGraw Hill, Inc., New York), which is incorporated by reference.

Atherosclerosis and myocardial infarction can lead to ischemia-reperfusion (I/R) injury. One of the underlying mechanisms for I/R-induced injury is the hypoxic and reoxygenated environments created in affected tissues. Fluctuations in oxygen content as observed in these instances can create oxygen free radicals which have been reported to, among other things, modulate endothelial cell surface profile.

Thus, in one aspect, the invention involves a method for treating or preventing myocardial infarction in a subject. "Myocardial infarction" is a focus of necrosis resulting from inadequate perfusion of the cardiac tissue. Myocardial infarction generally occurs with the abrupt decrease in coronary blood flow that follows a thrombotic occlusion of a coronary artery previously narrowed by atherosclerosis. Generally, infarction occurs when an atherosclerotic plaque fissures, ruptures, or ulcerates, and a mural thrombus forms leading to coronary artery occlusion.

A number of laboratory tests, well known in the art for diagnosis of myocardial infarction, are described, for example, in Harrison's: Principles of Internal Medicine (McGraw Hill, Inc., New York). Generally, the tests may be divided into four main categories: (1) nonspecific indexes of tissue necrosis and inflammation, (2) electrocardiograms, (3) serum enzyme changes (e.g., creatine phosphokinase levels), and (4) cardiac imaging. A person of ordinary skill in the art could easily apply any of the foregoing tests to determine when a subject is at risk, is suffering, or has suffered, a myocardial infarction. A positively identified subject would thus benefit from a method of treatment of the invention.

According to the invention, the method involves administering to a subject having or at risk of having a myocardial infarction an MBL receptor antagonist in an amount effective

to inhibit cardiac tissue necrosis in the subject. It is believed that immediate administration of an MBL receptor antagonist would greatly benefit the subject by inhibiting complement activation and associated tissue damage prior to, at the same time as, or following the infarct.

In one embodiment, when MBL receptor antagonists are used in the treatment of diseases (e.g., myocardial infarction), a growth factor may be co-administered. In some embodiments, Insulin-like Growth Factor-1 (IGF-1) is the growth factor of choice.

The co-administered growth factor or other medicament for the treatment of the MBL mediated disorder can act cooperatively, additively or synergistically with the MBL receptor antagonist of the invention to inhibit complement activation.

Optionally, in other embodiments of the invention for treating myocardial infarction, an isolated MBL receptor antagonist of the invention is administered to a subject in need of such treatment in combination with a method for treating an arteriosclerotic condition. An arteriosclerotic condition, as used herein, is a term of art that refers to classical atherosclerosis, accelerated atherosclerosis, atherosclerotic lesions and other physiological conditions characterized by undesirable vascular smooth muscle cell proliferation. See, e.g., Harrisons, Principles of Internal Medicine (McGraw Hill, Inc., New York) for a more detailed description of these conditions. The method for treating an arteriosclerotic condition may be a surgical method, an agent for treating restenosis, a method involving a drug therapy (e.g., gene therapy) or a combination of the foregoing.

Surgical methods for treating an arteriosclerotic condition include procedures such as bypass surgery, atherectomy, laser procedures, ultrasonic procedures, and balloon angioplasty. In a preferred embodiment of the invention, the isolated MBL receptor antagonist is administered to a subject in combination with a balloon angioplasty procedure. A balloon angioplasty procedure involves inserting a catheter having a deflated balloon into an artery. The deflated balloon is positioned in proximity to the atherosclerotic plaque and is inflated such that the plaque is compressed against the vascular wall. As a result, the balloon surface is in contact layer of vascular endothelial cells on the surface of the vessel. The isolated MBL receptor antagonist may be attached to the balloon angioplasty catheter in a manner which permits release of the isolated MBL receptor antagonist at the site of the atherosclerotic plaque. The isolated MBL receptor antagonist may be attached to the balloon angioplasty catheter in accordance with standard procedures known in the art. For example, the isolated MBL receptor antagonist may be stored in a compartment of the balloon

angioplasty catheter until the balloon is inflated, at which point it is released into the local environment. Alteratively, the isolated MBL receptor antagonist may be impregnated on the balloon surface, such that it contacts the cells of the arterial wall as the balloon is inflated. The MBL receptor antagonist also may be delivered in a perforated balloon catheter such as those disclosed in Flugelman, et al., *Circulation*, v. 85, p. 1110-1117 (1992). See, also, e.g., published PCT Patent Application WO 95/23161, for an exemplary procedure for attaching a therapeutic protein to a balloon angioplasty catheter. This procedure can be modified using no more that routine experimentation to attach a therapeutic nucleic acid or polypeptide to the balloon angioplasty catheter.

Additionally, the MBL receptor antagonist may be co-administered with a medicament for the treatment of the MBL mediated disease, e.g., anti-atherosclerotic agent for treating or preventing clinically significant atherosclerosis. The term "co-administered," means administered substantially simultaneously with another agent. By substantially simultaneously, it is meant that an MBL receptor antagonist of the invention is administered to the subject close enough in time with the administration of the other agent (e.g., an anti-atherosclerotic agent, growth factor, etc.).

Preferred anti-atherosclerotic agents used in combination with the MBL receptor antagonist of the invention, include but are not limited to, the following drugs: HMG-CoA reductase inhibitors, diuretics, antiadrenergic agents, vasodilators, calcium channel antagonists, angiotensin-converting enzyme (ACE) inhibitors, angiotensin II antagonists, and clot dissolvers.

"HMG-CoA reductase (3-hydroxy-3-methylglutaryl-coenzyme A)" is the microsomal enzyme that catalyzes the rate limiting reaction in cholesterol biosynthesis (HMG-CoA6Mevalonate). An "HMG-CoA reductase inhibitor" inhibits HMG-CoA reductase, and therefore inhibits the synthesis of cholesterol. There is a large number of compounds described in the art that have been obtained naturally or synthetically, which have been seen to inhibit HMG-CoA reductase, and which form the category of agents useful for practicing the present invention. Traditionally these agents have been used to treat individuals with hypercholesterolemia. Examples include some which are commercially available, such as simvastatin (U.S. Patent No. 4, 444,784), lovastatin (U.S. Patent No. 4,231,938), pravastatin sodium (U.S. Patent No. 4,346,227), fluvastatin (U.S. Patent No. 4,739,073), atorvastatin (U.S. Patent No. 5,273,995), cerivastatin, and numerous others

described in U.S. Patent No. 5,622,985, U.S. Patent No. 5,135,935, U.S. Patent No. 5,356,896, U.S. Patent No. 4,920,109, U.S. Patent No. 5,286,895, U.S. Patent No. 5,262,435, U.S. Patent No. 5,260,332, U.S. Patent No. 5,317,031, U.S. Patent No. 5,283,256, U.S. Patent No. 5,256,689, U.S. Patent No. 5,182,298, U.S. Patent No. 5,369,125, U.S. Patent No. 5,302,604, U.S. Patent No. 5,166,171, U.S. Patent No. 5,202,327, U.S. Patent No. 5,276,021, U.S. Patent No. 5,196,440, U.S. Patent No. 5,091,386, U.S. Patent No. 5,091,378, U.S. Patent No. 4,904,646, U.S. Patent No. 5,385,932, U.S. Patent No. 5,250,435, U.S. Patent No. 5,132,312, U.S. Patent No. 5,130,306, U.S. Patent No. 5,116,870, U.S. Patent No. 5,112,857, U.S. Patent No. 5,102,911, U.S. Patent No. 5,098,931, U.S. Patent No. 5,081,136, U.S. Patent No. 5,025,000, U.S. Patent No. 5,021,453, U.S. Patent No. 5,017,716, U.S. Patent No. 5,001,144, U.S. Patent No. 5,001,128, U.S. Patent No. 4,997,837, U.S. Patent No. 4,996,234, U.S. Patent No. 4,994,494, U.S. Patent No. 4,992,429, U.S. Patent No. 4,970,231, U.S. Patent No. 4,968,693, U.S. Patent No. 4,963,538, U.S. Patent No. 4,957,940, U.S. Patent No. 4,950,675, U.S. Patent No. 4,946,864, U.S. Patent No. 4,946,860, U.S. Patent No. 4,940,800, U.S. Patent No. 4,940,727, U.S. Patent No. 4,939,143, U.S. Patent No. 4,929,620, U.S. Patent No. 4,923,861, U.S. Patent No. 4,906,657, U.S. Patent No. 4,906,624 and U.S. Patent No. 4,897,402, the disclosures of which patents are incorporated herein by reference.

Diuretics include thiazides, e.g., hydrochlorothiazide; loop acting diuretics, e.g., furosemide; potassium-sparing, e.g., spironolactone, triamterene, and amiloride.

Antiadrenergic agents include clonidine; guanabenz; guanfacine; methyldopa; trimethapajn; Rauwolfia alkaloids, e.g., reserpine; guanethidine; guanadrel; phentolamine; phenoxybenzamine; prazosin; terazosin; propranolol; metoprolol; nadolol; atenolol; timolol; timdolol; acebutolol; and labetalol.

Vasodilators include hydralazine; minoxidil; diazoxide; and nitroprusside.

Calcium channel antagonists include nisadipine; diltiazen; and verapamil.

Angiotensin II antagonists are compounds which interfere with the activity of angiotensin II by binding to angiotensin II receptors and interfering with its activity. Angiotensin II antagonists are well known and include peptide compounds and non-peptide compounds. Most angiotensin II antagonists are slightly modified congeners in which agonist activity is attenuated by replacement of phenylalanine in position 8 with some other amino acid; stability can be enhanced by other replacements that slow degeneration *in vivo*. Examples of angiotensin II antagonists include: peptidic compounds (e.g., saralasin,

[(San^1)(Val^5)(Ala^8)] angiotensin -(1-8) octapeptide and related analogs); N-substituted imidazole-2-one (US Patent Number 5,087,634); imidazole acetate derivatives including 2-N-butyl-4-chloro-1-(2-chlorobenzile) imidazole-5-acetic acid (see Long et al., *J. Pharmacol. Exp. Ther.* 247(1), 1-7 (1988)); 4, 5, 6, 7-tetrahydro-1H-imidazo [4, 5-c] pyridine-6-carboxylic acid and analog derivatives (US Patent Number 4,816,463); N2-tetrazole beta-glucuronide analogs (US Patent Number 5,085,992); substituted pyrroles, pyrazoles, and tryazoles (US Patent Number 5,081,127); phenol and heterocyclic derivatives such as 1, 3-imidazoles (US Patent Number 5,073,566); imidazo-fused 7-member ring heterocycles (US Patent Number 5,064,825); peptides (e.g., US Patent Number 4,772,684); antibodies to angiotensin II (e.g., US Patent Number 4,302,386); and aralkyl imidazole compounds such as biphenyl-methyl substituted imidazoles (e.g., EP Number 253,310, January 20, 1988); ES8891 (N-morpholinoacetyl-(-1-naphthyl)-L-alanyl-(4, thiazolyl)-L-alanyl (35, 45)-4-amino-3-hydroxy-5-cyclo-hexapentanoyl-N-hexylamide, Sankyo Company, Ltd., Tokyo, Japan); SKF108566 (E-alpha-2-[2-butyl-1-(carboxy phenyl) methyl] 1H-imidazole-5-yl[methylane]-2-thiophenepropanoic acid, Smith Kline Beecham Pharmaceuticals, PA); Losartan (DUP753/MK954, DuPont Merck Pharmaceutical Company); Remikirin (RO42-5892, F. Hoffman LaRoche AG); A_2 agonists (Marion Merrill Dow) and certain non-peptide heterocycles (G.D.Searle and Company).

ACE, is an enzyme which catalyzes the conversion of angiotensin I to angiotensin II. ACE inhibitors include amino acids and derivatives thereof, peptides, including di- and tri-peptides and antibodies to ACE which intervene in the renin-angiotensin system by inhibiting the activity of ACE, thereby reducing or eliminating the formation of pressor substance angiotensin II. ACE inhibitors have been used medically to treat hypertension, congestive heart failure, myocardial infarction and renal disease. Classes of compounds known to be useful as ACE inhibitors include acylmercapto and mercaptoalkanoyl prolines such as captopril (U.S. Patent Number 4,105,776) and zofenopril (U.S. Patent Number 4,316,906), carboxyalkyl dipeptides such as enalapril (U.S. Patent Number 4,374,829), lisinopril (U.S. Patent Number 4,374,829), quinapril (U.S. Patent Number 4,344,949), ramipril (U.S. Patent Number 4,587,258), and perindopril (U.S. Patent Number 4,508,729), carboxyalkyl dipeptide mimics such as cilazapril (U.S. Patent Number 4,512,924) and benazapril (U.S. Patent Number 4,410,520), phosphinylalkanoyl prolines such as fosinopril (U.S. Patent Number 4,337,201) and trandolopril.

Renin inhibitors are compounds which interfere with the activity of renin. Renin inhibitors include amino acids and derivatives thereof, peptides and derivatives thereof, and antibodies to renin. Examples of renin inhibitors that are the subject of United States patents are as follows: urea derivatives of peptides (U.S. Patent Number 5,116,835); amino acids connected by nonpeptide bonds (U.S. Patent Number 5,114,937); di- and tri- peptide derivatives (U.S. Patent Number 5,106,835); amino acids and derivatives thereof (U.S. Patent Numbers 5,104,869 and 5,095,119); diol sulfonamides and sulfinyls (U.S. Patent Number 5,098,924); modified peptides (U.S. Patent Number 5,095,006); peptidyl beta-aminoacyl aminodiol carbamates (U.S. Patent Number 5,089,471); pyrolimidazolones (U.S. Patent Number 5,075,451); fluorine and chlorine statine or statone containing peptides (U.S. Patent Number 5,066,643); peptidyl amino diols (U.S. Patent Numbers 5,063,208 and 4,845,079); N-morpholino derivatives (U.S. Patent Number 5,055,466); pepstatin derivatives (U.S. Patent Number 4,980,283); N-heterocyclic alcohols (U.S. Patent Number 4,885,292); monoclonal antibodies to renin (U.S. Patent Number 4,780,401); and a variety of other peptides and analogs thereof (U.S. Patent Numbers 5,071,837, 5,064,965, 5,063,207, 5,036,054, 5,036,053, 5,034,512, and 4,894,437).

The invention also is useful for treating cellular injury arising from ischemia/reperfusion, e.g., associated with atherosclerosis and/or cardio-vascular remodeling. Injury to the vascular system can lead to a number of undesirable health conditions, including, for example, forms of atherosclerosis and arteriosclerosis that are associated with unwanted vascular smooth muscle cell proliferation. A common injury to the vascular system occurs as a side effect of a medical procedure for treating ischemic heart disease.

Ischemia refers to a lack of oxygen due to inadequate perfusion of blood. Ischemic heart disease is characterized by a disturbance in cardiac function due to an inadequate supply of oxygen to the heart. The most common form of this disease involves a reduction in the lumen of coronary arteries, which limits coronary blood-flow. Under these conditions the carbohydrate or peptide residues of the cell surface become exposed, the cells present a microbial carbohydrate (e.g., foam cell chlamydia), or an MBL ligand is synthesized, allowing MBL to associate with the cell surface and initiate the LCP associated complement activation.

When ischemic heart disease becomes very serious, then management must be invasive. Until recently, ischemic heart disease was treated by coronary-artery, bypass

surgery. Less invasive procedures, however, now have been developed. These procedures involve the use of catheters introduced into the narrowed region of the blood vessel ("the stenosis") for mechanically disrupting, laser ablating or dilating the stenosis.

An "ischemic disease or condition" as used herein refers to a condition characterized by local inflammation resulting from an interruption in the blood supply to a tissue due to a blockage or hemorrhage of the blood vessel responsible for supplying blood to the tissue such as is seen for myocardial or cerebral infarction. A cerebral ischemic attack or cerebral ischemia is a form of ischemic condition in which the blood supply to the brain is blocked. This interruption in the blood supply to the brain may result from a variety of causes, including an intrinsic blockage or occlusion of the blood vessel itself, a remotely originated source of occlusion, decreased perfusion pressure or increased blood viscosity resulting in inadequate cerebral blood flow, or a ruptured blood vessel in the subarachnoid space or intracerebral tissue.

The methods of the invention are useful also for treating cerebral ischemia. Cerebral ischemia may result in either transient or permanent deficits and the seriousness of the neurological damage in a patient who has experienced cerebral ischemia depends on the intensity and duration of the ischemic event. A transient ischemic attack is one in which the blood flow to the brain is interrupted only briefly and causes temporary neurological deficits, which often are clear in less than 24 hours. Symptoms of TIA include numbness or weakness of face or limbs, loss of the ability to speak clearly and/or to understand the speech of others, a loss of vision or dimness of vision, and a feeling of dizziness. Permanent cerebral ischemic attacks, also called stroke, are caused by a longer interruption in blood flow to the brain resulting from either a thromboembolism or hemorrhage. A stroke causes a loss of neurons typically resulting in a neurologic deficit that may improve but that does not entirely resolve. Thromboembolic stroke is due to the occlusion of an extracranial or intracranial blood vessel by a thrombus or embolus. Because it is often difficult to discern whether a stroke is caused by a thrombosis or an embolism, the term "thromboembolism" is used to cover strokes caused by either of these mechanisms. The term thromboembolism will be used throughout this patent application to describe thrombotic and embolic strokes. Hemorrhagic stroke is caused by the rupture of a blood vessel in a subarachnoid space or intracerebral tissue.

The methods of the invention in some embodiments are directed to the treatment of acute thromboembolic stroke. An acute stroke is a medical syndrome involving neurological

injury resulting from an ischemic event, which is an interruption in the blood supply to the brain. Acute stroke may be thromboembolic or hemorrhagic.

An effective amount of an MBL receptor antagonist alone or in combination with another therapeutic for the treatment of stroke is that amount sufficient to reduce *in vivo* brain injury resulting from the stroke. A reduction of brain injury is any prevention of injury to the brain which otherwise would have occurred in a subject experiencing a thromboembolic stroke absent the treatment of the invention. Several physiological parameters may be used to assess reduction of brain injury, including smaller infarct size, improved regional cerebral blood flow, and decreased intracranial pressure, for example, as compared to pretreatment patient parameters, untreated stroke patients or stroke patients treated with thrombolytic agents alone.

The pharmaceutical preparation of the MBL receptor antagonist also may be used alone or in combination with a therapeutic agent for treating an ischemic disease or condition. Therapeutics for treating ischemic diseases or conditions are described in medical textbooks such as Harrisons, Principles of Internal Medicine (McGraw Hill, Inc., New York). The particular therapeutic used depends on the nature of the disease or condition. Examples of therapeutics useful in the treatment of ischemic diseases or conditions include anticoagulation agents, antiplatelet agents, and thrombolytic agents.

Anticoagulation agents prevent the coagulation of blood components and thus prevent clot formation. Anticoagulants include, but are not limited to, heparin, warfarin, coumadin, dicumarol, phenprocoumon, acenocoumarol, ethyl biscoumacetate, and indandione derivatives.

Antiplatelet agents inhibit platelet aggregation and are often used to prevent thromboembolic stroke in patients who have experienced a transient ischemic attack or stroke. Antiplatelet agents include, but are not limited to, aspirin, thienopyridine derivatives such as ticlopodine and clopidogrel, dipyridamole and sulfinpyrazone, as well as RGD mimetics and also antithrombin agents such as, but not limited to, hirudin.

Thrombolytic agents lyse clots which cause the thromboembolic stroke. Thrombolytic agents have been used in the treatment of acute venous thromboembolism and pulmonary emboli and are well known in the art (e.g. see Hennekens et al, *J Am Coll Cardiol*; v. 25 (7 supp), p. 18S-22S (1995); Holmes, et al, *J Am Coll Cardiol*; v.25 (7 suppl), p. 10S-

17S(1995)). Thrombolytic agents include, but are not limited to, plasminogen, a_2-antiplasmin, streptokinase, antistreplase, tissue plasminogen activator (tPA), and urokinase.

In a preferred embodiment of the invention tPA is the thrombolytic agent. The mature tPA polypeptide has 527 amino acids, at least 17 (Asn) of which have been shown to be linked with carbohydrate structures. Spellman et al., have identified several of these carbohydrates, including a high-mannose structure on amino acid 117, and di-tri-and tetra-antennary N-acetyllactosamine-type structures on amino acids 184 and 448 *[J. Biol. Chem. 264* (24) 14100-14111 (1989)].

"tPA" as used herein includes native tPA and recombinant tPA, as well as modified forms of tPA that retain the enzymatic or fibrinolytic activities of native tPA. The enzymatic activity of tPA can be measured by assessing the ability of the molecule to convert plasminogen to plasmin. The fibrinolytic activity of tPA may be determined by any *in vitro* clot lysis activity known in the art, such as the purified clot lysis assay described by Carlson, et. al., *Anal. Biochem.* 168, 428-435 (1988) and its modified form described by Bennett, W. F. Et al., 1991, *Supra*, the entire contents of which are hereby incorporated by reference.

Recombinant tPA has been described extensively in the prior art. Several forms of recombinant tPA are commercially available such as ACTIVASE ®.

Modified forms of tPA ("modified tPA") have been characterized and are known to those skilled in the art. Modified tPA includes, but is not limited to, variants having deleted or substituted amino acids or domains, variants conjugated to other molecules, and variants having modified glycosylation. Several preferred modified tPAs have been described in PCT Publication No. W093/24635; EP 352,119; EP382174; and Suzuki et al., *J. Cardiovasc. Pharmacal. 22*, 834-840 (1993). Each of these references is hereby incorporated by reference.

Briefly, PCT Publication No. W093/24635 discloses tPA variants having an extra glycosylation site at any of the amino acid positions 103-105 and the native glycosylation site removed at position 117 of the native human tPA. The amino acid number refers to the amino acid in that position of the mature, wild-type tPA polypeptide as disclosed in US Pat. No. 4,766,075. These variants have extended circulatory half lives and exhibit substantially the same or improved fibrin binding affinity and fibrinolytic potency as compared to wild-type human tPA. The disclosed variants may also include at least one amino acid substituted in the 296-299 position with alanine and/or a substitution of the amino acids at positions 274-

277 of wild type tPA (phenylalanine, arginine, isoleucine, lysine) with leucine, histidine, serine, and threonine, respectively. One particularly effective type of variant disclosed in the reference is a triple mutant variant of wild type tPA. The first mutation in a triple mutant is the addition of one glycosylation site at least one of the amino acid positions 103-105 by e.g., substituting the native amino acid sequence 103 with an asparagine as part of an Asn-X-Ser or Asn-X-Thr tripeptidyl sequence, wherein X is any amino acid except proline. The second mutation involves the removal of a glycosylation site at native amino acid site 117 (Asn) and replacing it with another amino acid, preferably glutamine. The third mutation is the replacement of native amino acids 296-302 with other amino acids. The most effective of the triple mutant variants is the specific molecule, T103N, N117Q, KHRR (296-299) AAAA tPA (TNK tPA).

EP 352,119 discloses Vampire Bat tPA's (Bat-Pa (H), (I), and (L)). Vampire bat-Pa's are variants of native tPA having a variety of sequence modifications. Although the Bat-Pa variants are structurally distinct from tPA because they lack the Kringle 2 domain and plasmin-sensitive processing site, these variants are functionally similar to native tPA. They are however, more potent than native tPA.

Suzuki et al., *J. Cardiovasc. Pharmacal. 22,* 834-840 (1993) disclose tPA variants in which a cysteine at position 84 of the growth factor domain of native tPA is replaced by serine (C84S tPA). Although this variant retains the functional activity of native tPA, it has been shown to have a longer *in vivo* half life than native tPA.

The MBL receptor antagonists of the invention are directed to a specific epitope (e.g., present on endothelial cells) that mediates complement activation. Accordingly, the development of MBL receptor antagonists which inhibit MBL binding to this complement activating epitope on cells offers several advantages over inhibiting a specific complement component. First, selective inhibition of an endothelium epitope requires small amounts of MBL receptor antagonist to be given versus inhibiting a specific circulating complement component (e.g., most complement components circulate at concentrations in the range of 50 µg/ml of plasma and higher). Second, specific inhibition of ischemia/reperfusion induced complement activation at the epitope allows complement to be activated at distal sites for other host immune responses. Third, inhibition of a specific complement component may compromise the protective aspects of complement activation in other situations (e.g., opsonization). Thus, in view of the above-noted factors, selective inhibition of complement

at the site of complement activation is believed to be advantageous. See, Mulligan MS, Warner RL, Rittershaus CW, Thomas LJ, Ryan US, Foreman KE, Crouch LD, Till GO, Ward PA: Endothelial targeting and enhanced anti-inflammatory effects of complement inhibitors possessing sialyl Lewis moieties. *J. Immunol.* 1999;162:4952-4959, which reports that coupling of sCR1 with sialyl Lewis x provides site-directed inhibition of complement activation and tissue protection following ischemia/reperfusion injury by directing sCR1 to selectin ligands upregulated during ischemia/reperfusion injury.

The MBL receptor antagonists of the invention may be administered alone or in combination with other therapeutic treatments. For instance, the MBL receptor antagonist may be delivered with a medicament for the treatment of an MBL-mediated disorder.

The MBL receptor may be administered alone or may be delivered in a mixture with other medicaments, such as those disclosed herein and others known in the art. In some embodiments, a common administration vehicle (e.g., pill, tablet, implant, injectable solution, etc.) would contain both the MBL receptor antagonist useful in this invention and the therapeutic. Thus, the present invention also provides pharmaceutical compositions, for medical use, which comprise the MBL receptor antagonist of the invention together with one or more pharmaceutically acceptable carriers thereof and optionally other therapeutic ingredients.

An "MBL receptor antagonist" as used herein is a compound that prevents LCP-associated complement activation by inhibiting MBL binding to an MBL ligand (alternatively referred to herein as an "MBL receptor") expressed on the surface of a mammalian cell. The ability of an MBL receptor antagonist to block MBL deposition can be detected using routine *in vitro* binding assays, such as those described in the Examples as well as the following assay.

MBL deposition can be measured by ELISA on normoxic HUVECs and HUVECs subjected to 24 hr of hypoxia followed by 3 hr of reoxygenation in the presence of 30% human serum (HS) or 30% HS treated with 3, 30, or 300 mmol/L of N-acetyl-D-glucosamine (GlcNAc) or with the putative MBL receptor antagonist to inhibit competitively MBL deposition.

C3 and MBL specific cell surface ELISAs can be performed using peroxidase-conjugated polyclonal goat anti-human C3 antibody (Cappel, West Chester, PA) and monoclonal anti-human MBL antibody (Biodesign, Kennebunk, ME, clone #131-1),

respectively. HUVECs are grown to confluence on 0.1% gelatinized 96-well plastic plates (Corning Costar, Cambridge, MA). The plates are then subjected to 0 (normoxia) or 24 hr of hypoxia. Hypoxic stress is maintained using a humidified sealed chamber (Coy Laboratory Products, Inc., Grass Lake, MI) at 37 °C gassed with 1% O_2, 5% CO_2, balance N_2 (Collard CD, et al., "Reoxygenation of hypoxic human umbilical vein endothelial cells activates the classical complement pathway", *Circulation*, 1997;96:326-333). Following the specified period of normoxia or hypoxia, the cell media are aspirated and 100 µl of one the following is added to each well: 1) 30% HS, 2) Hank's balanced salt solution, 3) 30% HS + 3, 30, or 300 mmol/L GlcNAc, 4) 30% HS + 3, 30, or 300 mmol/L D-mannose, 5) 30% HS + 3, 30, 300 mmol/L L-mannose, 6) 30% MBL-depleted HS 7) 30% MBL-depleted HS + 0.6 µg/ml MBL or 8) 30% HS + 3, 30, or 300 mmol/L putative MBL binding peptide. Additionally, 100 µl of purified MBL (3-300 ng/ml) is added to select wells to form a standard curve for quantitative analysis of MBL deposition. The cells are then reoxygenated for 3 hr at 37°C in 95% air and 5% CO_2. The cells are washed and then fixed with 1% paraformaldehyde (Sigma Chemical Co., St. Louis, MO) for 30 min. The cells are then washed and incubated at 4 °C for 1.5 hr with 50 µl of peroxidase-conjugated polyclonal goat anti-human C3 antibody (1:1000 dilution) or monoclonal anti-human MBL antibody (1:1000 dilution). The MBL ELISA plates are then washed and incubated for 1 hr at 4 °C with 50 µl of peroxidase-conjugated polyclonal goat anti-mouse IgG secondary antibody (1:1000 dilution). After washing the cells, the plates are developed with 50 µl of ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)), and read (Molecular Devices, Sunnyvale, CA) at 405 nm. Background controls for the C3 ELISA consist of cells to which only the anti-human C3 antibody is added (i.e., no HS) or cells incubated with 30% heat-inactivated HS. Background controls for the MBL ELISA consist of cells to which only secondary antibody and an isotype control monoclonal antibody to porcine C5a are added. Background optical density is subtracted from all groups. All ELISA experiments are performed 3 times using 6 wells per experimental group. Endothelial C3 and MBL deposition on normoxic vs. hypoxic HUVECs is analyzed by two-way analysis of variance (ANOVA).

The MBL receptor antagonist prevents LCP-associated complement activation. Whether a particular compound can inhibit LCP-associated complement activation can also be assessed using routine *in vitro* screening assays. For instance, a complement hemolytic assay (CH_{50}) can be performed on MBL-depleted HS in order to demonstrate that MBL

depletion does not inhibit classical complement activation. The assay may be performed, however, using MBL containing HS and adding an MBL receptor antagonist and/or a control peptide to demonstrate specificity of the MBL inhibition.

In one illustrative embodiment, the MBL receptor antagonist is an isolated MBL binding peptide. An "isolated MBL receptor antagonist" as used herein is a peptide which binds to an MBL ligand ("MBL receptor") and inhibits LCP associated complement activation. It is believed that the MBL receptor antagonists inhibit LCP associated complement activation by binding to the MBL ligand (receptor) and inhibiting MBL association with surface exposed MBL ligands.

The preferred compositions of the invention include an MBL receptor antagonist which is an isolated binding molecule that selectively binds to a human MBL receptor and that inhibits LCP-associated complement activation. The MBL receptor antagonists of the invention can be identified using routine assays, such as the binding and LCP complement activation assays described above and elsewhere throughout this patent application.

The molecules of the invention are isolated molecules, e.g. isolated peptides. As used herein, with respect to molecules, the term "isolated molecules" means that the peptides are substantially pure and are essentially free of other substances with which they may be found in nature or *in vivo* systems to an extent practical and appropriate for their intended use. In particular, the molecules are sufficiently pure and are sufficiently free from other biological constituents of their hosts cells so as to be useful in, for example, producing pharmaceutical preparations or sequencing. Because an isolated molecule of the invention may be admixed with a pharmaceutically acceptable carrier in a pharmaceutical preparation, the molecule may comprise only a small percentage by weight of the preparation. The molecule is nonetheless substantially pure in that it has been substantially separated from the substances with which it may be associated in living systems. The term isolated refers to molecules which are either naturally occurring or synthetic. Thus, in some embodiments the isolated molecules are derived from natural sources.

In other embodiments the isolated molecules are synthetic MBL receptor antagonists may easily be synthesized or produced by recombinant means by those of skill in the art. Methods for preparing or identifying molecules which bind to a particular target are well known in the art. Molecular imprinting, for instance, may be used for the de novo construction of macromolecular structures such as peptides which bind to a particular

molecule. See for example Kenneth J. Shea, Molecular Imprinting of Synthetic Network Polymers: The De Novo synthesis of Macromolecular Binding and Catalytic Sites, *TRIP* Vol. 2, No. 5, May 1994; Klaus Mosbach, Molecular Imprinting, *Trends in Biochem. Sci.*, 19(9) January 1994; and Wulff, G., in Polymeric Reagents and Catalysts (Ford, W. T., Ed.) *ACS Symposium* Series No. 308, pp 186-230, *American Chemical Society* (1986). One method for preparing mimics of MBL receptor antagonists involves the steps of: (i) polymerization of functional monomers around a known MBL receptor antagonist that exhibits a desired activity; (ii) removal of the template molecule; and then (iii) polymerization of a second class of monomers in the void left by the template, to provide a new molecule which exhibits one or more desired properties which are similar to that of the template. In addition to preparing peptides in this manner other MBL receptor antagonists such as polysaccharides, nucleosides, drugs, nucleoproteins, lipoproteins, carbohydrates, glycoproteins, steroids, lipids, and other biologically active materials can also be prepared. This method is useful for designing a wide variety of biological mimics that are more stable than their natural counterparts, because they are typically prepared by the free radical polymerization of functional monomers, resulting in a compound with a nonbiodegradable backbone. Other methods for designing such molecules include for example drug design based on structure activity relationships which require the synthesis and evaluation of a number of compounds and molecular modeling.

Molecules which bind to the MBL receptor may also be identified by conventional screening methods such as phage display procedures (e.g., methods described in Hart, et al., *J. Biol. Chem.* 269:12468 (1994)). Hart et al. report a filamentous phage display library for identifying novel peptide ligands for mammalian cell receptors. In general, phage display libraries using, e.g., M13 or fd phage, are prepared using conventional procedures such as those described in the foregoing reference. The libraries display inserts containing from 4 to 80 amino acid residues. The inserts optionally represent a completely degenerate or a biased array of peptides. Receptor antagonists that bind selectively to MBL receptor are obtained by selecting those phage which express on their surface a peptide that binds to the MBL receptor. These phage then are subjected to several cycles of reselection to identify the peptide ligand-expressing phage that have the most useful binding characteristics. Typically, phage that exhibit the best binding characteristics (e.g., highest affinity) are further characterized by nucleic acid analysis to identify the particular amino acid sequences of the

peptides expressed on the phage surface and the optimum length of the expressed peptide to achieve optimum binding to the MBL receptor. Alternatively, such peptide ligands can be selected from combinatorial libraries of peptides containing one or more amino acids. Such libraries can further be synthesized which contain non-peptide synthetic moieties which are less subject to enzymatic degradation compared to their naturally-occurring counterparts.

Thus, according to another aspect of the invention, a method for optimizing a selected MBL receptor antagonist for its ability to bind to a MBL receptor and/or inhibit LCP-associated complement activation is provided. "Optimizing" as used herein refers to an iterative process of introducing changes to an existing system or compound and evaluating the functional significance of each change, followed by selecting the resulting system or compound associated with a functional outcome that is most improved; these steps are repeated until a desired endpoint is achieved or it appears further changes will not improve the functional outcome. The same objective can be achieved in a parallel manner by generating a library of closely related compounds and screening the library for the compound or compounds possessing the most favorable embodiment of the characteristic being optimized. In this particular instance, optimizing a selected MBL receptor antagonist for MBL receptor binding activity involves testing a panel of structurally related MBL receptor antagonists for their ability to bind to MBL receptor. The screening method involves contacting at least one candidate optimized MBL receptor antagonist selected from a group of candidate optimized MBL receptor antagonists with an MBL receptor under conditions which, in the absence of a competitor, permit a reference MBL receptor antagonist to bind or remain bound to the MBL receptor. The candidate optimized MBL receptor antagonist is contacted with the MBL receptor before, after, or simultaneously with contact between the labeled reference MBL receptor antagonist and the MBL receptor. The residual binding of the labeled reference MBL receptor antagonist to the MBL receptor is then detected. Detection of a decrease in binding of the reference MBL receptor antagonist indicates that the candidate optimized MBL receptor antagonist interferes with the binding of the reference MBL receptor antagonist to the MBL receptor. Candidate optimized MBL receptor antagonist can be generated as members of a combinatorial library of compounds, for example using SELEX technology. Gold L et al. (1995) *Annu Rev Biochem* 64:763:797.

This assay can involve the separation of both unbound unlabeled candidate optimized MBL receptor antagonists and unbound labeled reference MBL receptor antagonists from the

sample. The separation step can be accomplished in any way known in the art, in a manner similar to the separation method described above. Likewise, the detection of the remaining bound labeled reference MBL receptor antagonist can be accomplished in any way known in the art, in a manner similar to the detection method described above.

The screening assay can also be performed as a competition between labeled candidate optimized MBL receptor antagonists and unlabeled reference MBL receptor antagonists for the MBL receptor. In this format, binding of the labeled optimized MBL receptor antagonist to the MBL receptor is then detected. Detection of bound optimized MBL receptor antagonist indicates that the candidate optimized MBL receptor antagonist interferes with the binding of the reference MBL receptor antagonist to the MBL receptor.

The screening assay can also be performed by contacting labeled MBL receptor to immobilized MBL receptor antagonist. In this format a panel of candidate optimized MBL receptor antagonists can be presented in an array fashion on a silicon chip or in a plastic multiwell microtiter or microarray plate. Alternatively, each candidate optimized MBL receptor antagonist can be separately coupled to a bead, a resin, a nitrocellulose filter, a slide, or a biomolecular interaction analysis (BIA) chip. After contacting the MBL receptor with the immobilized candidate MBL receptor antagonists and, if indicated, washing away unbound MBL receptor, detection of complexes formed between the immobilized MBL receptor antagonist and the MBL receptor provides the basis for selecting particular MBL receptor antagonists as optimized. Exemplary molecular mimics of MBL which bind to an MBL receptor and/or inhibit LCP associated complement activation are provided in Example 7 (See, e.g., Table 1).

The above-described and other methods disclosed herein also can be used to identify molecular mimics of GlcNAc which bind to MBL. For example, the peptide referred to herein as "GLUPEP," potently inhibits iC3b deposition on HUVECS following oxidative stress in a dose-related manner. Accordingly, the screening and optimization methods disclosed herein can be used to identify molecular mimics of GLUPEP which bind to MBL and/or inhibit LCP associated complement activation. Exemplary molecular mimics of GlcNAc are provided in Example 1 (see, e.g., Table 2).

To determine whether a putative MBL receptor antagonist binds to an MBL receptor, any known binding assay may be employed. For example, the antagonist may be immobilized on a surface and then contacted with a labeled MBL receptor. The amount of

MBL receptor which interacts with the antagonist or the amount which does not bind to the antagonist may then be quantitated to determine whether the antagonist binds to the MBL receptor.

Screening of molecules of the invention, also can be carried out utilizing a competition assay. If the molecule being tested competes with MBL for binding to an MBL receptor, as shown by a decrease in binding of the MBL to the receptor, then it is likely that the molecule and the MBL bind to the same, or a closely related, epitope on the MBL receptor. Using routine procedures known to those of ordinary skill in the art, one can determine whether a molecule which binds to MBL receptor is useful according to the invention by determining whether the molecule is one which blocks MBL from binding to an MBL receptor. Such assays are described above and in the Examples section. Other assays will be apparent to those of skill in the art, having read the present specification, which are useful for determining whether a molecule which binds to MBL receptor also inhibits LCP associated complement activation.

Activation assays also can be used to assess the relative inhibitory concentrations of a molecule in an activation assay and to identify those molecules which inhibit activation by at least, e.g., 75%.

Other assays will be apparent to those of skill in the art, having read the present specification, which are useful for determining whether an MBL receptor antagonist which binds to an MBL receptor also inhibits MBL activation.

The procedures for the identification of a human MBL receptor expressed on endothelial cells are described in the Examples. The same strategy can be used to identify MBL receptors expressed in human and other cell types using no more than routine experimentation.

As discussed above the MBL receptor antagonists of the present invention encompass in some embodiments MBL receptor binding which include an MBL receptor binding region which specifically binds to a human MBL ligand and inhibits LCP associated complement activation, e.g., by preventing MBL from interacting with MBL ligands.

In certain embodiments, the MBL receptor antagonists are peptides which are derived from plant lectins. The MBL receptor antagonist is a legume derived lectin or a functional equivalent thereof that binds to the MBL ligand and that inhibits complement activation. A legume derived lectin is an isolated peptide (naturally occurring or synthetic) derived from a

legume. The legume derived lectin in some embodiments is *Ulex europaeus* (UEA)-II, *Laburnum alpinum* (LAA)-I, or Cytisus Sessilifolius anti-H(O) Lectin 1 (CSA-1). A functional equivalent of a legume derived lectin is a molecule, peptide or non-peptide that has an equivalent function to the legume derived lectins, such as a molecule having conservative substitutions.

As used herein, "conservative substitution" refers to an amino acid substitution which does not alter the relative charge or size characteristics of the peptide in which the amino acid substitution is made. Conservative substitutions of amino acids include substitutions made amongst amino acids with the following groups: (1) M,I,L,V; (2) F,Y,W; (3) K,R,H; (4) A,G; (5) S,T; (6) Q,N; and, (7) E,D. Such substitutions can be made by a variety of methods known to one of ordinary skill in the art to known MBL receptor antagonists of the invention to define novel peptide antagonists of the invention. For example, amino-acid substitutions may be made by PCR-directed mutation, site-directed mutagenesis according to the method of Kunkel (Kunkel, *Proc. Nat. Acad. Sci. U.S.A.* 82: 488-492, 1985), or by chemical synthesis of a gene encoding the CDR3 region. These and other methods are known to those of ordinary skill in the art and may be found in references which compile such methods, e.g. Sambrook. et al., *Molecular Cloning: A Laboratory Manual*, 2nd edition, Cold Spring Harbor Laboratory Press, 1989. The activity of functionally equivalent variants of the MBL receptor antagonists of the invention can be tested by the binding and activity assays discussed above.

As used herein, a "vector" may be any of a number of nucleic acids into which a desired sequence may be inserted by restriction and ligation for transport between different genetic environments or for expression in a host cell. Vectors are typically composed of DNA although RNA vectors are also available. Vectors include, but are not limited to, plasmids and phagemids. A cloning vector is one which is able to replicate in a host cell, and which is further characterized by one or more endonuclease restriction sites at which the vector may be cut in a determinable fashion and into which a desired DNA sequence may be ligated such that the new recombinant vector retains its ability to replicate in the host cell. In the case of plasmids, replication of the desired sequence may occur many times as the plasmid increases in copy number within the host bacterium or just a single time per host before the host reproduces by mitosis. In the case of phage, replication may occur actively during a lytic phase or passively during a lysogenic phase. An expression vector is one into which a desired DNA sequence may be inserted by restriction and ligation such that it is

operably joined to regulatory sequences and may be expressed as an RNA transcript. Vectors may further contain one or more marker sequences suitable for use in the identification of cells which have or have not been transformed or transfected with the vector. Markers include, for example, genes encoding proteins which increase or decrease either resistance or sensitivity to antibiotics or other compounds, genes which encode enzymes whose activities are detectable by standard assays known in the art (e.g., ß-galactosidase or alkaline phosphatase), and genes which visibly affect the phenotype of transformed or transfected cells, hosts, colonies or plaques. Preferred vectors are those capable of autonomous replication and expression of the structural gene products present in the DNA segments to which they are operably joined.

The expression vectors of the present invention include regulatory sequences operably joined to a nucleotide sequence encoding one of the peptides of the invention. As used herein, the term "regulatory sequences" means nucleotide sequences which are necessary for or conducive to the transcription of a nucleotide sequence which encodes a desired peptide and/or which are necessary for or conducive to the translation of the resulting transcript into the desired peptide. Regulatory sequences include, but are not limited to, 5' sequences such as operators, promoters and ribosome binding sequences, and 3' sequences such as polyadenylation signals. The vectors of the invention may optionally include 5' leader or signal sequences, 5' or 3' sequences encoding fusion products to aid in protein purification, and various markers which aid in the identification or selection of transformants. The choice and design of an appropriate vector is within the ability and discretion of one of ordinary skill in the art. The subsequent purification of the peptides may be accomplished by any of a variety of standard means known in the art.

A preferred vector for screening peptides, but not necessarily preferred for the mass production of the peptides of the invention, is a recombinant DNA molecule containing a nucleotide sequence that codes for and is capable of expressing a fusion peptide containing, in the direction of amino- to carboxy-terminus, (1) a prokaryotic secretion signal domain, (2) a peptide of the invention, and, optionally, (3) a fusion protein domain. The vector includes DNA regulatory sequences for expressing the fusion peptide, preferably prokaryotic regulatory sequences. Such vectors can be constructed by those with skill in the art and have been described by Smith et al. (*Science* 228:1315-1317, 1985), Clackson et al. (*Nature* 352:624-628, 1991); Kang et al. (in "Methods: A Companion to Methods in Enzymology:

Vol. 2", R.A. Lerner and D.R. Burton, ed. Academic Press, NY, pp 111-118,1991); Barbas et al. (*Proc. Natl. Acad. Sci. (USA)* 88:7978-7982, 1991), Roberts et al. (*Proc. Natl. Acad. Sci. (USA)* 89:2429-2433, 1992)

A fusion peptide may be useful for purification of the peptides of the invention. The fusion domain may, for example, include a poly-His tail which allows for purification on Ni+ columns or the maltose binding protein of the commercially available vector pMAL (New England BioLabs, Beverly, MA). A currently preferred, but by no means necessary, fusion domain is a filamentous phage membrane anchor. This domain is particularly useful for screening phage display libraries of monoclonal antibodies but may be of less utility for the mass production of antibodies. The filamentous phage membrane anchor is preferably a domain of the cpIII or cpVIII coat protein capable of associating with the matrix of a filamentous phage particle, thereby incorporating the fusion peptide onto the phage surface, to enable solid phase binding to specific antigens or epitopes and thereby allow enrichment and selection of the binding peptides or fragments encoded by the phagemid vector.

The secretion signal is a leader peptide domain of a protein that targets the protein membrane of the host cell, such as the periplasmic membrane of gram negative bacteria. A preferred secretion signal for *E. coli* is a pelB secretion signal. The predicted amino acid residue sequences of the secretion signal domain from two pelB gene producing variants from *Erwinia carotova* are described in Lei, et al. (*Nature* 381:543-546, 1988). The leader sequence of the pelB protein has previously been used as a secretion signal for fusion proteins (Better, et al., *Science* 240:1041-1043, 1988; Sastry, et al., *Proc. Natl. Acad. Sci (USA)* 86:5728-5732, 1989; and Mullinax, et al., *Proc. Natl. Acad. Sci. (USA)* 87:8095-8099, 1990). Amino acid residue sequences for other secretion signal peptide domains from *E. coli* useful in this invention can be found in Oliver, In Neidhard, F.C. (ed.), *Escherichia coli and Salmonella Typhimurium*, American Society for Microbiology, Washington, D.C., 1:56-69 (1987).

To achieve high levels of gene expression in *E. coli*, it is necessary to use not only strong promoters to generate large quantities of mRNA, but also ribosome binding sites to ensure that the mRNA is efficiently translated. In *E. coli*, the ribosome binding site includes an initiation codon (AUG) and a sequence 3-9 nucleotides long located 3-11 nucleotides upstream from the initiation codon (Shine, et al., *Nature* 254:34, 1975). The sequence, AGGAGGU, which is called the Shine-Dalgarno (SD) sequence, is complementary to the 3'

end of *E. coli* 16S rRNA. Binding of the ribosome to mRNA and the sequence at the 3' end of the mRNA can be affected by several factors:

(i) The degree of complementarity between the SD sequence and 3' end of the 16S rRNA.

(ii) The spacing and possibly the DNA sequence lying between the SD sequence and the AUG (Roberts, et al., *Proc. Natl. Acad. Sci. (USA)* 76:760.,1979a: Roberts, et al., *Proc. Natl. Acad. Sci. (USA)* 76:5596, 1979b; Guarente, et al., *Science* 209:1428, 1980; and Guarente, et al., *Cell* 20:543, 1980). Optimization is achieved by measuring the level of expression of genes in plasmids in which this spacing is systematically altered. Comparison of different mRNAs shows that there are statistically preferred sequences from positions -20 to +13 (where the A of the AUG is position 0) (Gold, et al., *Annu. Rev. Microbiol.* 35:365, 1981). Leader sequences have been shown to influence translation dramatically (Roberts, et al., 1979a, b supra).

(iii) The nucleotide sequence following the AUG, which affects ribosome binding (Taniguchi, et al., *J. Mol. Biol.*, 118:533, 1978).

The 3' regulatory sequences define at least one termination (stop) codon in frame with and operably joined to the heterologous fusion peptide.

In preferred embodiments with a prokaryotic expression host, the vector utilized includes a prokaryotic origin of replication or replicon, i.e., a DNA sequence having the ability to direct autonomous replication and maintenance of the recombinant DNA molecule extra-chromosomally in a prokaryotic host cell, such as a bacterial host cell, transformed therewith. Such origins of replication are well known in the art. Preferred origins of replication are those that are efficient in the host organism. A preferred host cell is *E. coli*. For use of a vector in *E. coli*, a preferred origin of replication is ColE1 found in pBR322 and a variety of other common plasmids. Also preferred is the p15A origin of replication found on pACYC and its derivatives. The ColE1 and p15A replicons have been extensively utilized in molecular biology, are available on a variety of plasmids and are described by Sambrook. et al., *Molecular Cloning: A Laboratory Manual*, 2nd edition, Cold Spring Harbor Laboratory Press, 1989).

In addition, those embodiments that include a prokaryotic replicon preferably also include a gene whose expression confers a selective advantage, such as drug resistance, to a

bacterial host transformed therewith. Typical bacterial drug resistance genes are those that confer resistance to ampicillin, tetracycline, neomycin/kanamycin or chloramphenicol. Vectors typically also contain convenient restriction sites for insertion of translatable DNA sequences. Exemplary vectors are the plasmids pUC18 and pUC19 and derived vectors such as pcDNAII available from Invitrogen, (San Diego, CA).

The receptor antagonist peptides of the present invention may also, of course, be produced by eukaryotic cells such as CHO cells, human hybridomas, immortalized B-lymphoblastoid cells, and the like. In this case, a vector is constructed in which eukaryotic regulatory sequences are operably joined to the nucleotide sequences encoding the peptide. The design and selection of an appropriate eukaryotic vector is within the ability and discretion of one of ordinary skill in the art. The subsequent purification of the peptides may be accomplished by any of a variety of standard means known in the art.

In another embodiment, the present invention provides host cells, both prokaryotic and eukaryotic, transformed or transfected with, and therefore including, the vectors of the present invention.

As used herein, a coding sequence and regulatory sequences are said to be "operably joined" when they are covalently linked in such a way as to place the expression or transcription of the coding sequence under the influence or control of the regulatory sequences. If it is desired that the coding sequences be translated into a functional peptide, two DNA sequences are said to be operably joined if induction of a promoter in the 5' regulatory sequences results in the transcription of the coding sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the coding sequences, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a promoter region would be operably joined to a coding sequence if the promoter region were capable of effecting transcription of that DNA sequence such that the resulting transcript might be translated into the desired peptide.

The precise nature of the regulatory sequences needed for gene expression may vary between species or cell types, but shall in general include, as necessary, 5' non-transcribing and 5' non-translating sequences involved with initiation of transcription and translation respectively, such as a TATA box, capping sequence, CAAT sequence, and the like.

Especially, such 5' non-transcribing regulatory sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably joined gene. Regulatory sequences may also include enhancer sequences or upstream activator sequences, as desired.

In other embodiments the MBL receptor antagonist is a keratin binding molecule. A keratin binding molecules may be isolated from natural sources or synthesized or produced by recombinant means. Methods for preparing or identifying molecules which bind to a particular target are well-known in the art and are described above. Binding peptides, such as antibodies, may easily be prepared by generating antibodies to keratin (or obtained from commercial sources) or by screening libraries to identify peptides or other compounds which bind to the keratin.

Many keratin antibodies are commercially available. These include but are not limited to those antibodies commercially available from Research Diagnostics, Inc., e.g., RDI-CYTOK1abr (Cytokeratin 1 rabbit polyclonal); RDI- (mouse cytokeratins rabbit polyclonals); RDI-CBL222 (Cytokeratin 1-3 AE8 + mIgG1); RDI-PRO61808 (Cytokeratin 1,10/11 K8.60 + mIgG1); RDI-PRO65177 (Cytokeratin 2E Ks 2.398.3.1 mIgG1); RDI-PRO65191 (Cytokeratin 2E Ks2.342.7.1 + mIgG1); RDI-CBL218 (Cytokeratin 3 (bovine & rabbit) AE5 mIgG1); RDI-PRO10525 (Cytokeratin 4 (most mammals) 6B10 mIgG1); RDI-CBL234 (Cytokeratin 4,5,6,8,10,13,18 C11 mIgG); RDI-CBL232 (Cytokeratin 5,8 C50 + mIgG1); RDI-PRO10521 (Cytokeratin 5+8 RCK102 + mIgG1); RDI-PRO61531 (Cytokeratin 5+8 RCK12+ Biotin conj); RDI-PRO61431 (Cytokeratin 5+8 RCK102+ FITC conj); RDI-PRO61031 (Cytokeratin 5+8(pan epithelial) C22 mIgG1); RDI-PRO65190 (Cytokeratin 6 Ks6.Ka12+ mIgG1); RDI-CBL194 (Cytokeratin 7 LP5K mIgG2b); RDI-CBL184 (Cytokeratin 7 Lds68 IgM); RDI-PRO61025 (Cytokeratin 7 (bovine, sheep, pig) KS7.18+ mIgG1); RDI-PRO10522 (Cytokeratin 7 (hamster, mouse) RCK105 mIgG1); RDI-CBL195 (Cytokeratin 8 LP3K mIgG1); RDI-CBL195FT (Cytokeratin 8 LP3K mIgG1 FITC); RDI-PRO61038 (Cytokeratin 8 (mouse, rat, pig, hamster) Ks8.7+* mIgG1); RDI-PRO65130 (Cytokeratin 8(phosphorylated) Ks 8-17.2 mIgG1); RDI-CBL170 (Cytokeratin 8,18 5D3 + mIgG2a); RDI-PRO10526 (Cytokeratin 8 (most mammals) M20 mIgG1); RDI-PRO651104 (Cytokeratin 9 KS9.7 & KS 9.216 mIgG1/mIgG3); RDI-CBL196 (Cytokeratin 10 LH2 mIgG); RDI-PRO10501 (Cytokeratin 10 (rat, mouse, bovine, rabbit) RKSE60 mIgG1); RDI-PRO11414 (Cytokeratin 10 DE-K10 + mIgG1); RDI-CBL217 (Cytokeratin 10,11,1 & 2 AE2

+ mIgG1); RDI-PRO61007 (Cytokeratin 13 (bovine & rat) Ks13.1 + mIgG1); RDI-PRO10523 (Cytokeratin 13 (human, mouse, rabbit) 1C7 mIgG2a); RDI-PRO10524 (Cytokeratin 13 (human, mouse, rabbit) 2D7 mIgG2b); RDI-CBL197 (Cytokeratin 14 LL002 + mIgG3); RDI-CBL197FT (Cytokeratin 14 LL002 + mIgG3 FITC); RDI-PRO10003 (Cytokeratin 14 RCK 107 mIgG1); RDI-PRO61036 (Cytokeratin 17 (rat 46kDa polyp) KS 17.E3+ mIgG2b); RDI-CBL236 (Cytokeratin 18 CO4 + mIgG1); RDI-PRO61028 (Cytokeratin 18 (mouse, rat, pig, dog, sheep, hamster, bovine, trout) Ks18.04 + mIgG1); ARDI-PRO11416 (Cytokeratin 18 RCK 106 mIgG1); RDI-PRO10500 (Cytokeratin 18 RGE 53 mIgG1); RDI-CBL185 (Cytokeratin 18 DC10 + mIgG1); RDI-CBL178 (Cytokeratin 19 Ks19.1 + mIgG2a); RDI-CBL198 (Cytokeratin 19 BA17 + mIgG1); RDI-PRO61029 (Cytokeratin 19 (rat, fish, bovine) KS 19.2 mIgG2b); RDI-CBL199 (Cytokeratin 19 LAS86 mIgM); RDI-CBL247 (Cytokeratin 19 A53-B/A2.26 mIgG2a); RDI-CBL208 (Cytokeratin 20 Ks20.8 + mIgG1); RDI-PRO61054 (Cytokeratin 20 (rat, pig) mouse IT-KS20.10 + mIgG1); RDI-PRO61033 (Cytokeratin 20 Ks20.5 mIgG2a); RDI-CBL215 (Cytokeratin Type I (monkey, rabbit, mouse, rat, bovine, chicken, monkey) AE1 + mIgG1); RDI-CBL216 (Cytokeratin Type II monkey, rabbit, mouse, rat, bovine, chicken, monkey) AE3 + mIgG1); RDI-PRO61031 (Cytokeratin (Pan epithelial) C22 mIgG1); RDI-PRO61006 (Cytokeratin TYPE II Ks pan 1-8 mIgG2a); RDI-PRO61056 (Cytokeratin TYPE II " " Biotin labeled); RDI-PRO61406 (Cytokeratin Type II " " FITC conjugated); and RDI-PRO61835 (Cytokeratin Type I & II AE1/AE3 mIgG1). These antibodies also include anti-pan-cytokeratin (human, bovine rat and mouse, catalog number 250400) from CALBIOCHEM, and product numbers C7034 (anti-cytokeratin 8.12); C6909 (anti-cytokeratin 8.13); C7284 (anti-cytokeratin 8.60); C7785 (anti-cytokeratin CK5); C8791 (anti-cytokeratin peptide 14); and C1399 (anti-cytokeratin peptide 18) from Sigma-Aldrich.

Thus, as described above, a template, such as a keratin binding antibody can be used to identify keratin binding molecules. It is now routine to produce large numbers of molecules having inhibitory functions based on one or a few peptide sequences or sequence motifs. (See, e.g., Bromme, et al., *Biochem. J.* 315:85-89 (1996); Palmer, et al., *J. Med. Chem.* 38:3193-3196 (1995)). For example, if keratin is known to interact with protein X (i.e. MBL) at position Y, an inhibitor of keratin-MBL interactions may be chosen or designed as a polypeptide or modified polypeptide having the same sequence as protein X, or structural similarity to the sequence of protein X, in the region adjacent to position Y. In fact, the

region adjacent to the cleavage site Y spanning residues removed by 10 residues or, more preferably 5 residues, N-terminal and C-terminal of position Y, may be defined as a "preferred protein X site" for the choice or design of keratin-MBL interaction inhibitors. Thus, a plurality of these compounds chosen or designed to span the preferred protein X binding site around position Y, may be produced, tested for inhibitory activity, and sequentially modified to optimize or alter activity, stability, and/or specificity.

The method is useful for designing a wide variety of biological mimics that are more stable than the natural counterpart, because they are typically prepared by the free radical polymerization of functional monomers, resulting in a compound with a non-biodegradable backbone. Thus, the created molecules would have the same binding properties as the keratin antibody but be more stable *in vivo*, thus preventing keratin from interacting with components normally available in its native environment. Other methods for designing such molecules include, for example, drug design based on structure activity relationships which require the synthesis and evaluation of a number of compounds and molecular modeling.

Binding molecules may also be identified by conventional screening methods, such as those described above. Additionally, keratin binding molecules can be identified from combinatorial libraries. Many types of combinatorial libraries have been described. For instance, U.S. Patent Nos. 5,712,171 (which describes methods for constructing arrays of synthetic molecular constructs by forming a plurality of molecular constructs having the scaffold backbone of the chemical molecule and modifying at least one location on the molecule in a logically-ordered array); 5, 962, 412 (which describes methods for making polymers having specific physiochemical properties); and 5, 962, 736 (which describes specific arrayed compounds).

By using the known keratin monoclonal antibodies, it is also possible to produce anti-idiotypic antibodies which can be used to screen other antibodies to identify whether the antibody has the same binding specificity as the known monoclonal antibody. Such anti-idiotypic antibodies can be produced using well-known hybridoma techniques (*Kohler and Milstein, Nature, 256:495, 1975*). An anti-idiotypic antibody is an antibody which recognizes unique determinants present on the known monoclonal antibodies. These determinants are located in the hypervariable region of the antibody. It is this region which binds to a given epitope and, thus, is responsible for the specificity of the antibody. An anti-idiotypic antibody can be prepared by immunizing an animal with the known monoclonal

antibodies. The immunized animal will recognize and respond to the idiotypic determinants of the immunizing known monoclonal antibodies and produce an antibody to these idiotypic determinants. By using the anti-idiotypic antibodies of the immunized animal, which are specific for the known monoclonal antibodies, it is possible to identify other clones with the same idiotype as the known monoclonal antibody used for immunization. Idiotypic identity between monoclonal antibodies of two cell lines demonstrates that the two monoclonal antibodies are the same with respect to their recognition of the same epitopic determinant. Thus, by using anti-idiotypic antibodies, it is possible to identify other hybridomas expressing monoclonal antibodies having the same epitopic specificity.

It is also possible to use the anti-idiotype technology to produce monoclonal antibodies which mimic an epitope. For example, an anti-idiotypic monoclonal antibody made to a first monoclonal antibody will have a binding domain in the hypervariable region which is the image of the epitope bound by the first monoclonal antibody.

In one embodiment the binding molecules useful according to the invention are antibodies or functionally active antibody fragments. Antibodies are well known to those of ordinary skill in the science of immunology. Many of the binding molecules described herein are available from commercial sources as intact functional antibodies, as described above. As used herein, the term "antibody" means not only intact antibody molecules but also fragments of antibody molecules retaining specific binding ability. Such fragments are also well known in the art and are regularly employed both *in vitro* and *in vivo*. In particular, as used herein, the term "antibody" means not only intact immunoglobulin molecules but also the well-known active fragments $F(ab')_2$, and Fab. $F(ab')_2$, and Fab fragments which lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding of an intact antibody (*Wahl et al., J. Nucl. Med. 24:316-325 (1983)*).

As is well-known in the art, the complementarity determining regions (CDRs) of an antibody are the portions of the antibody which are largely responsible for antibody specificity. The CDR's directly interact with the epitope of the antigen (see, in general, *Clark, 1986; Roitt, 1991*). In both the heavy chain and the light chain variable regions of IgG immunoglobulins, there are four framework regions (FR1 through FR4) separated respectively by three complementarity determining regions (CDR1 through CDR3). The framework regions (FRs) maintain the tertiary structure of the paratope, which is the portion

of the antibody which is involved in the interaction with the antigen. The CDRs, and in particular the CDR3 regions, and more particularly the heavy chain CDR3 contribute to antibody specificity. Because these CDR regions and in particular the CDR3 region confer antigen specificity on the antibody these regions may be incorporated into other antibodies or peptides to confer the identical specificity onto that antibody or molecule.

According to one embodiment, the molecule is an intact soluble monoclonal antibody in an isolated form or in a pharmaceutical preparation. An intact soluble monoclonal antibody, as is well known in the art, is an assembly of polypeptide chains linked by disulfide bridges. Two principle polypeptide chains, referred to as the light chain and heavy chain, make up all major structural classes (isotypes) of antibody. Both heavy chains and light chains are further divided into subregions referred to as variable regions and constant regions. As used herein the term "monoclonal antibody" refers to a homogenous population of immunoglobulins which specifically bind to an epitope (i.e. antigenic determinant) , e.g., of keratin.

The molecule useful according to the methods of the present invention may be an intact humanized a monoclonal antibody. A "humanized monoclonal antibody" as used herein is a human monoclonal antibody or functionally active fragment thereof having human constant regions and a binding CDR3 region from a mammal of a species other than a human. Humanized monoclonal antibodies may be made by any method known in the art. Humanized monoclonal antibodies, for example, may be constructed by replacing the non-CDR regions of a non-human mammalian antibody with similar regions of human antibodies while retaining the epitopic specificity of the original antibody. For example, non-human CDRs and optionally some of the framework regions may be covalently joined to human FR and/or Fc/pFc' regions to produce a functional antibody. There are entities in the United States which will synthesize humanized antibodies from specific murine antibody regions commercially, such as Protein Design Labs (Mountain View California). For instance, a humanized form of the anti-keratin antibody used in the attached Examples could be easily prepared and used according to the methods of the invention.

European Patent Application 0239400, the entire contents of which is hereby incorporated by reference, provides an exemplary teaching of the production and use of humanized monoclonal antibodies in which at least the CDR portion of a murine (or other non-human mammal) antibody is included in the humanized antibody. Briefly, the following

methods are useful for constructing a humanized CDR monoclonal antibody including at least a portion of a mouse CDR. A first replicable expression vector including a suitable promoter operably linked to a DNA sequence encoding at least a variable domain of an Ig heavy or light chain and the variable domain comprising framework regions from a human antibody and a CDR region of a murine antibody is prepared. Optionally a second replicable expression vector is prepared which includes a suitable promoter operably linked to a DNA sequence encoding at least the variable domain of a complementary human Ig light or heavy chain respectively. A cell line is then transformed with the vectors. Preferably the cell line is an immortalized mammalian cell line of lymphoid origin, such as a myeloma, hybridoma, trioma, or quadroma cell line, or is a normal lymphoid cell which has been immortalized by transformation with a virus. The transformed cell line is then cultured under conditions known to those of skill in the art to produce the humanized antibody.

As set forth in European Patent Application 0239400 several techniques are well known in the art for creating the particular antibody domains to be inserted into the replicable vector. (Preferred vectors and recombinant techniques are discussed in greater detail below.) For example, the DNA sequence encoding the domain may be prepared by oligonucleotide synthesis. Alternatively a synthetic gene lacking the CDR regions in which four framework regions are fused together with suitable restriction sites at the junctions, such that double stranded synthetic or restricted subcloned CDR cassettes with sticky ends could be ligated at the junctions of the framework regions. Another method involves the preparation of the DNA sequence encoding the variable CDR containing domain by oligonucleotide site-directed mutagenesis. Each of these methods is well known in the art. Therefore, those skilled in the art may construct humanized antibodies containing a murine CDR region without destroying the specificity of the antibody for its epitope.

Human monoclonal antibodies may be made by any of the methods known in the art, such as those disclosed in US Patent No. 5,567,610, issued to Borrebaeck et al., US Patent No. 565,354, issued to Ostberg, US Patent No. 5,571,893, issued to Baker et al, Kozber, J. Immunol. 133: 3001 (1984), Brodeur, et al., Monoclonal Antibody Production Techniques and Applications, p. 51-63 (Marcel Dekker, Inc, new York, 1987), and Boerner et al., J. Immunol., 147: 86-95 (1991). In addition to the conventional methods for preparing human monoclonal antibodies, such antibodies may also be prepared by immunizing transgenic animals that are capable of producing human antibodies (e.g., Jakobovits et al., PNAS USA,

90: 2551 (1993), Jakobovits et al., Nature, 362: 255-258 (1993), Bruggermann et al., Year in Immuno., 7:33 (1993) and US Patent No. 5,569,825 issued to Lonberg).

The binding peptides may also be functionally active antibody fragments. Significantly, as is well-known in the art, only a small portion of an antibody molecule, the paratope, is involved in the binding of the antibody to its epitope (see, in general, Clark, W.R. (1986) The Experimental Foundations of Modern Immunology Wiley & Sons, Inc., New York; Roitt, I. (1991) Essential Immunology, 7th Ed., Blackwell Scientific Publications, Oxford). The pFc' and Fc regions of the antibody, for example, are effectors of the complement cascade but are not involved in antigen binding. An antibody from which the pFc' region has been enzymatically cleaved, or which has been produced without the pFc' region, designated an $F(ab')_2$ fragment, retains both of the antigen binding sites of an intact antibody. An isolated $F(ab')_2$ fragment is referred to as a bivalent monoclonal fragment because of its two antigen binding sites. Similarly, an antibody from which the Fc region has been enzymatically cleaved, or which has been produced without the Fc region, designated an Fab fragment, retains one of the antigen binding sites of an intact antibody molecule. Proceeding further, Fab fragments consist of a covalently bound antibody light chain and a portion of the antibody heavy chain denoted Fd (heavy chain variable region). The Fd fragments are the major determinant of antibody specificity (a single Fd fragment may be associated with up to ten different light chains without altering antibody specificity) and Fd fragments retain epitope-binding ability in isolation.

The terms Fab, Fc, pFc', $F(ab')_2$ and Fv are used consistently with their standard immunological meanings [Klein, *Immunology* (John Wiley, New York, NY, 1982); Clark, W.R. (1986) *The Experimental Foundations of Modern Immunology* (Wiley & Sons, Inc., New York); Roitt, I. (1991) *Essential Immunology*, 7th Ed., (Blackwell Scientific Publications, Oxford)].

According to the methods of the invention, the compositions may be administered in a pharmaceutically acceptable composition. In general, pharmaceutically-acceptable carriers for peptides and structurally-related small molecules are well-known to those of ordinary skill in the art. As used herein, a pharmaceutically-acceptable carrier means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients, i.e., the ability of the MBL receptor antagonist to inhibit LCP associated complement activation. Pharmaceutically acceptable carriers include diluents, fillers, salts,

buffers, stabilizers, solubilizers and other materials which are well-known in the art. Exemplary pharmaceutically acceptable carriers for peptides in particular are described in U.S. Patent No. 5,211,657. The receptor antagonist peptides of the invention may be formulated into preparations in solid, semi-solid, liquid or gaseous forms such as tablets, capsules, powders, granules, ointments, solutions, depositories, inhalants (e.g., aerosols) and injections, and usual ways for oral, parenteral or surgical administration. The invention also embraces locally administering the compositions of the invention, including as implants.

According to the methods of the invention the compositions can be administered by injection by gradual infusion over time or by any other medically acceptable mode. The administration may, for example, be intravenous, intraperitoneal, intramuscular, intracavity, subcutaneous or transdermal. Preparations for parenteral administration includes sterile aqueous or nonaqueous solutions, suspensions and emulsions. Examples of nonaqueous solvents are propylene glycol, polyethylene glycol, vegetable oil such as olive oil, an injectable organic esters such as ethyloliate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers, (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, antioxidants, chelating agents, and inert gases and the like. Those of skill in the art can readily determine the various parameters for preparing these alternative pharmaceutical compositions without resort to undue experimentation. When the compositions of the invention are administered for the treatment of pulmonary disorders the compositions may be delivered for example by aerosol.

The compositions of the invention are administered in therapeutically effective amounts. As used herein, an "effective amount" of the inhibitor of the invention is a dosage which is sufficient to inhibit the increase in, maintain or even reduce the amount of undesirable LCP associated complement activation. The effective amount is sufficient to produce the desired effect of inhibiting associated cellular injury until the symptoms associated with the MBL mediated disorder are ameliorated or decreased. Preferably an effective amount of the peptide is an effective amount for preventing cellular injury. Generally, a therapeutically effective amount may vary with the subject's age, condition, and

sex, as well as the extent of the disease in the subject and can be determined by one of skill in the art. The dosage may be adjusted by the individual physician or veterinarian in the event of any complication. A therapeutically effective amount typically will vary from about 0.01 mg/kg to about 500 mg/kg, were typically from about 0.1 mg/kg to about 200 mg/kg, and often from about 0.2 mg/kg to about 20 mg/kg, in one or more dose administrations daily, for one or several days (depending of course of the mode of administration and the factors discussed above). A preferred concentration of the MBL receptor antagonist is a concentration which is equimolar to the concentration of MBL in the plasma of a subject. The normal plasma concentration of MBL can be assessed clinically. A normal range of MBL is 1-2mg/L MBL/plasma.

One of skill in the art can determine what an effective amount of an MBL receptor antagonist is by screening the ability of the antagonist to inhibit the LCP associated complement activation in an *in vitro* assay. The activity of the antagonist can be defined in terms of the ability of the antagonist to inhibit LCP associated complement activation. An exemplary assay for measuring the ability of a putative MBL receptor antagonist of the invention to inhibit LCP associated complement activation is provided in the Examples and has been discussed above. The exemplary assay is predictive of the ability of an MBL receptor antagonist to inhibit LCP associated complement activation *in vivo* and, hence, can be used to select such antagonists for therapeutic applications.

The MBL receptor antagonists may be administered in a physiologically acceptable carrier. The term "physiologically-acceptable" refers to a non-toxic material that is compatible with the biological systems of a tissue or organism. The physiologically acceptable carrier must be sterile for *in vivo* administration. The characteristics of the carrier will depend on the route of administration. The characteristics of the carrier will depend on the route of administration.

The invention further provides detectably labeled, immobilized and toxin conjugated forms of the peptides of the invention, as well as fragments and functional equivalents thereof. The MBL receptor antagonists of the invention may be labeled using radiolabels, fluorescent labels, enzyme labels, free radical labels, avidin-biotin labels, or bacteriophage labels, using techniques known to the art (Chard, *Laboratory Techniques in Biology*, "An Introduction to Radioimmunoassay and Related Techniques," North Holland Publishing Company (1978).

Typical fluorescent labels include fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, and fluorescamine.

Typical chemiluminescent compounds include luminol, isoluminol, aromatic acridinium esters, imidazoles, and the oxalate esters.

Typical bioluminescent compounds include luciferin, and luciferase. Typical enzymes include alkaline phosphatase, ß-galactosidase, glucose-6-phosphate dehydrogenase, maleate dehydrogenase, glucose oxidase, and peroxidase.

The invention also includes methods for screening a subject for susceptibility to treatment with an MBL receptor antagonist. In one aspect, the method is accomplished by isolating a mammalian cell from a subject and detecting the presence of an MBL or an MBL ligand on a surface of the mammalian cell. The presence of the MBL indicates that the cell is susceptible to LCP-associated complement activiation, and that the subject is susceptible to treatment with an MBL receptor antagonist. The mammalian cell may be isolated by any method known in the art, for instance by a biopsy. Another method for accomplishing the screening assay involves the steps of contacting a mammalian cell from the subject with a labeled isolated MBL receptor antagonist and detecting the presence of an MBL receptor antagonist the surface of the mammalian cell. This assay may be performed *in vitro*, *ex vivo*, or *in vivo*. Many labels which can be used to observe the MBL receptor antagonist interacting with the mammalian cell are known in the art under each of these conditions. For instance, radioactive compounds can be used *in vitro*, and other biocompatible labels can be used *ex vivo* or *in vivo*. Once the subjects are identified who are susceptible to treatment with an MBL receptor antagonist, the subjects can then be treated according to the methods of the invention.

The following examples are provided to illustrate specific instances of the practice of the present invention and are not to be construed as limiting the present invention to these examples. As will be apparent to one of ordinary skill in the art, the present invention will find application in a variety of compositions and methods. The following abbreviations have been used throughout the Examples: MBL=mannose binding lectin; K=cytokeratin; CRD=carbohydrate recognition domain; MASP=MBL associated serine protease; PMN=polymorphonuclear leukocytes; mAb=monoclonal antibody; HUVECs=human umbilical vein endothelial cells; GlcNAc=N-acetylglucosamine; ROS=reactive oxygen species; HS=human serum/sera; I/R=ischemia/reperfusion; H/R=hypoxia/reoxygenation;

$NF_{\kappa}B$=nuclear factor κ B; AA=amino acid; UEA= *Ulex europaeus*; LAA= *Laburnum alpinum*; DEF=desferrioxamine; DMTU=dimethylthiourea.

Examples

Introduction to the Examples:

The experiments described herein investigate the molecular mechanisms of complement activation during or following ischemia/reperfusion and are useful for developing novel therapeutics to inhibit complement activation during reperfusion. More particularly, the experiments disclosed herein 1) evaluate novel legume-based complement inhibitors directed against an endothelial MBL ligand; 2) describe structure/activity relationships of novel functionally inhibitory peptides that block MBL binding to endothelial cells following oxidative stress; 3) characterize the regulation of endothelial cell K expression during oxidative stress; and 4) characterize the action and/or the mechanism of UEA-II induced tissue protection following ischemia/reperfusion injury *in vivo*.

Example 1. The Role of MBL in activating complement on hypoxic/reoxygenated endothelial cells.

Previous published findings from our lab demonstrate that reoxygenation/ reperfusion of hypoxic/ischemic endothelial cells generates intracellular reactive oxygen species which activate $NF\text{-}_{\kappa}B$, leading to transcription and translation of a novel ligand for MBL (Collard CD, Agah A, Stahl GL: Complement activation following reoxygenation of hypoxic human endothelial cells: role of intracellular reactive oxygen species, NF- kappaB and new protein synthesis. *Immunopharmacology* 1998;39:39-50). The intracellular response to hypoxia/ reoxygenation leads to upregulation of a ligand(s) for MBL, complement activation and iC3b/C5b-9 deposition on the extracellular membrane. We have developed a panel of mAbs that bind to the CRD of MBL and thus functionally inhibit MBL binding to the endothelial MBL ligand. (USSN 60/112,390, entitled "Anti-Mannose (Mannan) Binding Lectin Therapy for Ischemia/Reperfusion Injury.") A significant increase in C3 and MBL deposition on endothelial cells was observed following oxidative stress (in the presence of 30% HS) compared to normoxic cells. Treatment of the human sera with a non-functionally inhibitory mAb (clone 1C10; 50 μg/ml) to MBL did not attenuate MBL or C3 deposition. In contrast, clone 3F8 (5 μg/ml) significantly attenuated MBL and C3 deposition. Co-localization of

MBL and C3 was also demonstrated in this study. These data coupled with other data demonstrating that depletion of MBL from sera, treatment with a specific MBL inhibitory sugar (i.e., N-acetylglucosamine) and use of purified complement components conclusively demonstrated that MBL is responsible for complement activation on endothelial cells following oxidative stress.

Example 2. Characterization of the MBL ligand present on endothelial cells following oxidative stress.

Recognition and characterization of a MBL ligand and generation of functionally inhibitory molecules to the ligand represent a novel therapeutic approach for complement inhibition. The principal binding ligands of MBL are oligosaccharide oligomers, particularly oligomers of GlcNAc and mannose. We believe that the novel epitope for MBL binding on hypoxic/reoxygenation HUVECs is a glycoprotein (Collard CD, Agah A, Stahl GL: Complement activation following reoxygenation of hypoxic human endothelial cells: role of intracellular reactive oxygen species, NF- kappaB and new protein synthesis. *Immunopharmacology* 1998;39:39-50). Since MBL purification is tedious and the yield from 1 L of human plasma varies from 100-200 μg, immunoprecipitation of the ligand with purified MBL would be very costly and time consuming. We therefore developed another strategy. There are many legume lectins that display "MBL-like" binding characteristics (e.g., calcium-dependent and inhibited by specific sugars). Thus, we screened the binding characteristics of several known legume lectins that are competitively inhibited by GlcNAc or complex oligomers of GlcNAc and display calcium-dependent binding. After screening eleven different lectins, the lectin *Ulex europaeus* II(UEA-II) showed an increase in deposition on hypoxic (1% O_2; 24 hrs)/ reoxygenated (3 hrs, room air, in buffer) HUVECs compared to normoxic cells. Deposition of UEA-II to hypoxic/reoxygenated cells was similar to that of MBL as it: 1) was inhibited by DMTU treatment; 2) was inhibited by DEF, but not iron-loaded DEF, 3) displayed calcium-dependent binding, and 4) was inhibited by cycloheximide treatment (n=3 for each group; data not shown). Further, when UEA-II was bound to hypoxic HUVECs, MBL competitively inhibited UEA-II binding in a dose-dependent manner. Similarly, MBL binding to H/R HUVECs was competitively inhibited by UEA-II in a dose-dependent manner. Thus, it appeared that UEA-II and MBL bound to the same ligand.

UEA-II (CY Laboratories) was coupled to Sepharose. Cell membranes were prepared by nitrogen cavitation from 1500 cm^2 of either normoxic or hypoxic/reoxygenated HUVECs. The membranes were solubilized and pre-cleared with Sepharose. UEA-II coupled Sepharose was used for immunoprecipitation. The resulting immunoprecipitate was resolved under reducing conditions by 9% SDS-PAGE. A single Coomassie stainable diffuse band (MW ~49-54 kDa) was cut from the gel and sent to our Core Facility (Bill Lane; Harvard MicroChemistry) for tryptic digestion and MALDI-TOF MS analysis. A peptide (QIEGLKEELAYLR/K, SEQ ID NO. 1) was sequenced that displayed a high degree of homology to human K14, K15, K16, K17 and K19. In order to demonstrate that the peptide was present on the cell surface, we biotinylated the cell surface proteins, immunoprecipitated with UEA-II and the immunoprecipitate was resolved under reducing conditions by 9% SDS-PAGE. The gel was electroblotted onto nitrocellulose, the membrane blocked and resolved with HRP-streptavidin. HRP-streptavidin was visualized with the ECL system (Amersham). A single band (~50 kDa) from H/R cells was observed that was not present on normoxic cells. The approximate MW of 50 kDa is consistent with the known MW of K14 and K17, but not K15, K16 or K19. Searching the literature we found that anti-endothelial antibodies from patients with accelerated transplant coronary artery disease recognize a 50 kDa keratin-like protein on human endothelial cells (Ationu A: Identification of endothelial antigens relevant to transplant coronary artery disease from a human endothelial cell cDNA expression library. *Int.J.Mol.Med.* 1998;1:1007-1010). Further, a previous publication demonstrated that a peptide AA sequence within K14 (SFGSGFGGGY, SEQ ID NO. 2) mimics N-acetylglucosamine (GlcNAc) in reactions with anti-N-acetylglucosamine antibodies and some plant lectins(Shikhman AR, Greenspan NS, Cunningham MW: Cytokeratin peptide SFGSGFGGGY, SEQ ID NO. 2 mimics N-acetyl-beta-D- glucosamine in reaction with antibodies and lectins, and induces in vivo anti-carbohydrate antibody response. *J.Immunol.* 1994;153:5593-5606). This was an important finding, as GlcNAc is a potent inhibitor of MBL when it is present in the fluid phase. However, a BLAST search demonstrated that this AA sequence (i.e., SFGSGFGGGY, SEQ ID NO. 2) is also present in K17, CK1 and similar sequences are present in many cytokeratins. If this AA sequence does indeed mimic N-acetylglucosamine (i.e., a specific ligand of MBL), then MBL may be binding to this specific AA sequence of K14 or K17. We had the SFGSGFGGGY, SEQ ID NO. 2 peptide synthesized (referred to herein as "GLUPEP"). GLUPEP potently inhibits iC3b deposition

on HUVECs following oxidative stress in a dose-related manner. These data are consistent with the hypothesis that cytokeratins are responsible for MBL deposition and complement activation following oxidative stress. It is well recognized in the literature that MBL binds to carbohydrate groups, however this is the first demonstration of a peptide inhibiting the MBL pathway.

To verify that keratin will activate complement via the MBL pathway, we developed two solid phase ELISAs for the functional characterization of MBL and its associated serine-proteases (i.e., MASP1 and MASP2) based on a published manuscript(Super M, Levinsky RJ, Turner MW: The level of mannan-binding protein regulates the binding of complement-derived opsonins to mannan and zymosan at low serum concentrations. *Clin.Exp.Immunol.* 1990;79:144-150). In these assays, keratin or GLUPEP coupled to BSA was coated onto 96 well microtiter RIA/EIA plates. Human serum (2%) was then added to the wells, incubated for 30 min, the plates are washed and HRP-conjugated anti-C3 polyclonal antibodies was used to assess C3 deposition. Positive and negative controls consist of wells receiving vehicle and GlcNAc (100 mmol/L) or our functionally inhibitory mAb to hMBL (clone 3F8, deposited with the ATCC on December 15, 1998 under Accession number HB-126621), respectively. As shown in figure 2, keratin coated plates activated complement and deposited C3 that was significantly inhibited by either GlcNAc or 3F8 (i.e., MBL pathway activation). Further, GLUPEP or UEA-II inhibited keratin-induced complement activation in a dose-dependent manner. When GLUPEP was coupled to BSA, we demonstrated that MBL bound to GLUPEP-BSA, but not to BSA and that GlcNAc, 3F8, or GLUPEP inhibited MBL binding (Figure 3). These data demonstrate that keratin activates the MBL pathway and that MBL binds to GLUPEP. Based upon these results, we believe that purified K activates the MBL pathway. In view of this discovery, we believe that novel peptide inhibitors of the MBL pathway can be designed since previous results have reported that structure/activity relationships of GLUPEP, as specific substitutions of the AA sequence have been increased affinity of anti-GlcNAc antibodies to GLUPEP by as much as 350% (Shikhman AR, Greenspan NS, Cunningham MW: Cytokeratin peptide SFGSGFGGGY, SEQ ID NO. 2 mimics N-acetyl-beta-D- glucosamine in reaction with antibodies and lectins, and induces in vivo anti-carbohydrate antibody response. *J.Immunol.* 1994;153:5593-5606).

Example 3. Oxidative Stress Increases Human Endothelial Cell Surface CK1 protein Expression

A significant increase in endothelial cell surface CK1 expression following oxidative stress was observed. The data are consistent with the hypothesis that MBL may bind to many different keratins since the amino and hydroxy terminals of cytokeratins are highly homologous. Interestingly, exons 1 and 9 of CK1 contain sequences highly homologous to the amino acid sequence SFGSGFGGGY, SEQ ID NO. 2 which is present in K17.

Human endothelial cell CK1 protein expression following oxidative stress was determined by both ELISA and confocal microscopy. ELISA measurements indicate a significant increase in HUVEC CK1 expression following oxidative stress was observed as compared to the normoxic HUVEC (OD_{405} = 0.43 ± 0.05 vs 0.14 ± 0.01, respectively; $p<0.05$). Immunofluorescent confocal microscopy studies confirmed that oxidative stress caused a significant increase in extracellular HUVEC CK1 expression. The immunofluorescent experiments were performed three times (n=3).

In order to demonstrate the specificity of the anti-human CK1 pAb used in these experiments, HUVEC CK1 was immunoprecipitated (67 kDa band, reduced 9% linear SDS-PAGE gel) and confirmed by protein sequencing (human keratin, Type II cytoskeletal 1).

Example 4. Purified Human CK1 Activates the LCP

Endothelial cytokeratins induce complement activation following oxidative stress. Human MBL and C3 deposition on purified human dermal CK1 was determined by ELISA. Treatment of 2% HS with GlcNAc (100 mmol/L Sigma) or the functionally inhibitory anti-human MBL mAB, 3F8 (10 µg/ml) significantly inhibited MBL deposition on purified CK1-coated plates by 78 ± 4% and 64 ± 6%, respectively compared to untreated HS (vehicle). Further, treatment with GlcNAc (100 mmol/L) or 3F8 (10 µg/ml) significantly inhibited C3 deposition on CK1-coated plates by 70 ± 1% and 69 ± 1%, respectively compared to untreated HS. (n=3; data normalized to vehicle).

These data suggest that MBL binds human CK1 and activates the complement pathway. Furthermore, these data demonstrate that MBL inhibition attenuates CK1-induced complement activation.

Example 5. Human MBL Recognizes Endothelial CK1 Following Oxidative Stress.

In order to determine whether human MBL binds endothelial CK1, purified human MBL was used to immunoprecipitate HUVEC CK1. Western blot of the immunoprecipitates using a monospecific anti-human CK1 antibody revealed a 67-kDa band consistent with human CK1. Interestingly, the 67-kDa band was observed following endothelial oxidative stress, but not in normoxic HUVEC or in the control lanes. These data strongly suggest that human MBL recognizes and binds to endothelial CK1 following oxidative stress.

To further confirm that human MBL binds endothelial CK1 following oxidative stress, MBL and CK1 were co-immunoprecipitated from hypoxic HUVEC reoxygenated in HS. Western blot of HUVEC lysates immunoprecipitated with a monospecific anti-human CK1 pAb Convance/BAbCO (Richmond, CA) revealed a 32-kDa band consistent with reduced purified human MBL. Further, the 32-kDa band was observed following endothelial oxidative stress, but not in normoxic HUVEC or in the control lanes.

The data that MBL binds endothelial CK1 are consistent with the earlier studies indicating that MBL may bind other cytokeratins. As already noted earlier, exons 1 and 9 of CK1 contain sequences highly homologous to the amino acid sequence SFGSGFGGGY, SEQ ID NO. 2 which is present in K17 and other cytokeratins.

Example 6. Anti-human Keratin Treatment Attenuates MBL and C3 Deposition Following Endothelial Oxidative Stress.

Oxidative stress increases MBL deposition on HUVEC and activates the lectin complement pathway. In the present set of experiments it is demonstrated that anti-human keratin antibodies attenuate endothelial MBL and C3 deposition.

HUVEC MBL and C3 deposition following oxidative stress were measured by ELISA. Consistent with the previous findings, a significant increase in MBL (OD_{405} = 0.05 ± 0.01) and C3 (OD_{405} = 0.21 ± 0.02) deposition was observed following oxidative stress compared to normaxic HUVEC (OD_{405} = 0.01 ± 0.01 and 0.07 ± 0.01, respectively; $p<0.05$). Treatment of 30% HS with GlcNAc (100 mmol/L) or anti-human keratin pAb (50 μg/ml) significantly inhibited MBL deposition by 66 ± 11% and 53 ± 9%, respectively, compared to untreated HS (vehicle). Further, treatment with GlcNAc (100 mmol/L) or anti-human keratin Fab fragments (20 μg/ml synthesized in Stahl laboratory) significantly inhibited C3 deposition by 46 ± 7% and 48 ± 6%, respectively, compared to untreated HS. These data

demonstrate that anti-MBL or keratin treatment significantly attenuates endothelial MBL and C3 deposition following oxidative stress.

To further confirm these findings, HUVEC MBL and C3 deposition following oxidative stress was determined by immunofluorescent confocal microscopy. Normoxic and hypoxic HUVEC were reoxygenated in 30% HS treated with and without anti-human keratin Fab fragments (20 μg/ml). Small amounts of MBL and C3 staining were observed under normoxic conditions, confirming our previous finding of low level C3 deposition under normoxic conditions. MBL and C3 staining following HUVEC oxidative stress was increased compared to normoxic cells. Incubation of human sera with anti-human keratin Fab fragments attenuated MBL and C3 staining. These data further demonstrate that anti-keratin treatment inhibits MBL deposition and complement activation following endothelial oxidative stress.

Example 7. UEA-II Inhibits C3 Deposition on Keratin.

As shown above, UEA-II recognizes a MBL ligand on human endothelial cells following oxidative stress. We hypothesized that UEA-II may also inhibit complement activation by acting as a "receptor/ligand antagonist". Confocal micrographs of HUVECs following oxidative stress (in the presence of 30% HS) demonstrated co-localization of MBL and C3. We observed complete inhibition of C3 and MBL deposition at a UEA-II concentration of 100 pmol/L (10 ng/ml). Significant reductions in C3 and MBL deposition were also observed at 100 fmol/L (10 pg/ml) and 0.10 fmol/L (10 fg/ml). These data demonstrate that UEA-II is a potent complement inhibitor on endothelial cells following oxidative stress.

An experiment was performed in which keratin (2 μg/ml) or BSA (2 μg/ml) in sodium carbonate buffer was plated to 96-well plastic plates. The plates were then blocked with BSA. Human sera (30%) was applied to the wells in the presence of GVB (vehicle), GlcNAc (100 mmol/L) or UEA-II (E-Y laboratories, Manson, CA) (10 ng/ml). The plates were incubated at 37C for 1 hour, washed and incubated with a HRP-anti-human C3 Pab (YCN, Aurora, Ohio) for detection of C3 deposition to the plastic. Both GlcNAc or UEA-II inhibited the activation of the LCP and the resultant C3 deposition to the keratin coated plastic. These data further demonstrate that keratin does indeed activate the lectin complement pathway. The results are shown in Figure 5.

It was possible that UEA-II inhibited complement activation by activating complement and thus produced complement-depleted sera. In order to rule this out, UEA-II at 100 μg/ml (i.e., ~1 μmol/L) was incubated with human sera and hemolytic assays using sensitized chicken RBCs were performed. UEA-II did not activate complement or attenuate classical pathway activation. Thus, the mechanism of UEA-II mediated complement inhibition on HUVECs following oxidative stress is not a result of complement depletion, but a result of specific inhibition of MBL binding to its ligand. Further, UEA-II deposition on H/R HUVECs is inhibited by GLUPEP. These data demonstrate that GLUPEP inhibits UEA-II deposition on HUVECs following oxidative stress or MBL-dependent complement activation on keratin (Figure 2). Since GLUPEP inhibits MBL and UEA-II deposition, these data suggest that GLUPEP mimics GlcNAc.

The lectins UEA-II, *Cytisus sessilifolius* (CSA-I) and *Laburnum alpinum* (LAA-I) share a similar CRD peptide sequence (Yamamoto K, Konami Y, Osawa T, Irimura T: Carbohydrate-binding peptides from several anti-H(O) lectins. *J.Biochem.(Tokyo.)* 1992;111:436-439) (see Table 1). Like UEA-II, LAA-I inhibited MBL mediated C3 deposition on HUVECs following oxidative stress in a dose-dependent manner (Figure 4). Other lectins (e.g., CSA-I and LAA-I, and functional equivalents thereof such as fragments and/or peptides containing conservative amino acid substitutions) can be evaluated for inhibiting MBL binding to keratin and H/R HUVECs using the methods disclosed herein.

Table 1. Amino acid sequences for the CRD of legume lectins.

CSA-I: DTYFGKTYNPW, SEQ ID NO. 3

LAA-I: DTYFGKAYNPW, SEQ ID NO. 4

UEA-II: DSYFGKTYNPW, SEQ ID NO. 5

Derived from a published manuscript (Yamamoto K, Konami Y, Osawa T, Irimura T: Carbohydrate-binding peptides from several anti-H(O) lectins. *J.Biochem.(Tokyo.)* 1992;111:436-439). Highlighted amino acids represent divergent amino acids.

We have demonstrated that activation of the MBL pathway mediates the complement activation and deposition on human endothelial cells following oxidative stress and have developed antibody technology to inhibit MBL directly (USSN 60/112,390, entitled "Anti-

Mannose (Mannan) Binding Lectin Therapy for Ischemia/Reperfusion Injury). We disclose herein the discoveries of an MBL ligand present on human endothelial cells and that MBL, which is known to bind carbohydrate groups, binds to specific peptides in the absence of carbohydrate groups. Thus, the further experiments presented below describe the further investigation of the role of cytokeratin in activating the MBL pathway. These experiments also are directed to developing and conducting studies relating to structure/activity relationships of novel potent peptide based MBL inhibitors based on the MBL recognition of cytokeratin and the development of specific functionally inhibitory mAbs/Fabs to cytokeratin in order to inhibit MBL deposition and lectin pathway activation.

Example 8. Characterization of K expression following oxidative stress.

The above-described data strongly supports the upregulation of keratins and particularly CK1 as a novel MBL ligand on hypoxic/reoxygenated HUVECs. The experiments disclosed herein evaluate the expression of other keratins following oxidative stress to assess their role in the physiological results of oxidative stress (similar ro the studies performed on CK1). A recent report demonstrated that anti-endothelial antibodies from patients with accelerated transplant coronary artery disease recognize a 50 kDa keratin-like protein on human endothelial cells (Ationu A: Identification of endothelial antigens relevant to transplant coronary artery disease from a human endothelial cell cDNA expression library. *Int.J.Mol.Med.* 1998;1:1007-1010). Data from our lab has demonstrated that intracellular ROS, new protein synthesis and NFκB translocation is necessary for the MBL ligand to be upregulated. The experiments presented herein establish that K is a ligand for MBL on HUVECs following oxidative stress.

Several strategies are used to identify the specific keratin present on HUVECs. First, we have designed specific primers to K17 and K14 based on their cDNA and conserved regions using commercially available software (Sequencer). RT-PCR, northern and RNAse protection assays are performed to establish an increase in mRNA levels in HUVECs following oxidative stress using K specific probes. These data establish that mRNA is present and upregulated by oxidative stress. Oxidative stress is induced by H/R and also by H_2O_2, as we have described above. A time course for K expression is done. Full length cDNAs or a cell line (i.e., T84 or HeLa cells) is used as a positive control for all experiments. The RT-

PCR products for K14 in HUVECs are cloned/sequenced and all positive RT-PCR products are sequenced to demonstrate specificity.

Rabbits and mice are immunized for Pab and mAb production against GLUPEP. Antibodies to keratins are made and purified. Cell surface proteins are immunoprecipitated from HUVECs following normoxic or oxidative stress conditions using our monospecific Pab to GLUPEP. This immunoprecipitate recovers proteins containing this AA sequence. A western blot is performed using the commercially available monospecific Pab to K14 (BAbCo) or a mAb to K17 (clone E3; Dako) to determine which K is present. These experiments establish which K is present on the HUVECs. Additionally, cell surface proteins are biotinylated on HUVECs and immunoprecipitate with commercially available mAbs and detected which K is present on the cell surface with streptavidin-HRP.

In situ hybridization and confocal microscopy is performed to demonstrate localization of K to endothelial cells as we have described previously (Collard CD, Bukusoglu C, Agah A, Colgan SP, Reenstra WR, Morgan BP, Stahl GL: Hypoxia-induced expression of complement receptor type 1 (CR1, CD35) in human vascular endothelial cells. *Am.J.Physiol.* 1999;276:C450-C458). Localization of the K protein to the cell surface also is confirmed by the use of antibodies and confocal microscopy.

We have identified 3 potential cAMP response elements binding (CREB) sites in the 5' promoter region of K17, based on a BLAST search of the published promoter region of K17. Further, a previous study has demonstrated that K17 expression can be enhanced by γ-interferon under the direct activation of the transcription factor STAT1 (Komine M, Freedberg IM, Blumenberg M: Regulation of epidermal expression of keratin K17 in inflammatory skin diseases. *J.Invest.Dermatol.* 1996;107:569-575). However, preliminary data demonstrate that γ-interferon does not induce K17 in HUVECs by RT-PCR. Since hypoxia is known to induce CREB (Taylor CT, Fueki N, Agah A, Hershberg RM, Colgan SP: Critical Role of cAMP Response Element Binding Protein Expression in Hypoxia-elicited Induction of Epithelial Tumor Necrosis Factor-alpha. *J.Biol.Chem.* 1999;274:19447-19454), the following experiments are performed to investigate K17 expression. We have shown that complement activation is decreased in the presence of intracellular ROS production and NFκB translocation. As STAT1 activation is associated with ROS and increased K expression (Komine M, Freedberg IM, Blumenberg M: Regulation of epidermal expression of keratin K17 in inflammatory skin diseases. *J.Invest.Dermatol.* 1996;107:569-575), K

protein expression is evaluated under oxidative stress conditions known to increase complement activation. HUVECs undergo H/R and/or H_2O_2 (1-1000 μmol/L) are given to HUVECs as previously described (Collard CD, Agah A, Stahl GL: Complement activation following reoxygenation of hypoxic human endothelial cells: role of intracellular reactive oxygen species, NF- kappaB and new protein synthesis. *Immunopharmacology* 1998;39:39-50). NFκB translocation is inhibited by administration of SN50 (SM50 as a control), using NFκB decoys (Kupatt C, Habazettl H, Goedecke A, Wolf DA, Zahler S, Boekstegers P, Kelly RA, Becker BF: Tumor necrosis factor-alpha contributes to ischemia- and reperfusion-induced endothelial activation in isolated hearts. *Circ.Res.* 1999;84:392-400), or MG132 (proteasome inhibitor). A cell permeable cAMP stable analogue (i.e., dinitro-cAMP) or forskolin is used to pretreat endothelial cells prior to hypoxia to prevent CRE phosphorylation and formation of the CREB/CPB complex and resulting transcription. Protein phosphorylation events are known to regulate K17 expression in keratinocytes (Komine M, Freedberg IM, Blumenberg M: Regulation of epidermal expression of keratin K17 in inflammatory skin diseases. *J.Invest.Dermatol.* 1996;107:569-575). Thus, staurosporine and genistein should inhibit, whereas okadaic acid should augment, K expression.

The above-described experiments are used to identify K as the MBL ligand, and that K expression is enhanced by hypoxia/reoxygenation. We expect to observe an increase in K mRNA and protein expression following oxidative stress. These experiments lay the groundwork for understanding K regulation in HUVECs. The actions of pro-inflammatory cytokines and K regulation also are investigated. It is possible that the K present on HUVECs is a K-like, 50 kDa protein as previously described (Ationu A: Identification of endothelial antigens relevant to transplant coronary artery disease from a human endothelial cell cDNA expression library. *Int.J.Mol.Med.* 1998;1:1007-1010). With the tools we have developed (antibodies), immunoprecipitation is performed using the anti-GLUPEP antibodies and the protein is sequenced (e.g., the Harvard MicroChemistry Core facility for micro-sequencing) to confirm its identity.

Example 9. Development and characterization of small molecular weight inhibitors of MBL based on its interaction site with cytokeratin (Structure/activity relationship analysis)

Our preliminary data support an important role for K in mediating complement activation and MBL binding to HUVECs following oxidative stress. UEA-II and GLUPEP inhibit MBL deposition and the resulting complement activation in several assays, as shown above. A previous study has shown that specific AA substitutions within GLUPEP increase and decrease the affinity of anti-GlcNAc antibodies and lectins to the new peptides (Shikhman AR, Greenspan NS, Cunningham MW: Cytokeratin peptide SFGSGFGGGY, SEQ ID NO. 2 mimics N-acetyl-beta-D- glucosamine in reaction with antibodies and lectins, and induces in vivo anti-carbohydrate antibody response. *J.Immunol.* 1994;153:5593-5606). Our data are the first to demonstrate that MBL interacts with peptides or proteins in the absence of carbohydrate groups. The following prophetic experiments describe the evaluation and characterization of the actions of these peptides on MBL binding, MBL inhibition and complement activation. The data collected and analyzed in this section are useful for establishing the rank order potency of each peptide. These data are then used to evaluate the energy minimization of these conformations. These data are then used to aid in the development of small molecular weight inhibitors based on total organic synthesis.

A determination of the structure/activity relationship for GLUPEP to interact and inhibit MBL is performed as follows. A previous study has shown that specific AA substitutions within GLUPEP increase and decrease the affinity of anti-GlcNAc antibodies and lectins (Shikhman AR, Greenspan NS, Cunningham MW: Cytokeratin peptide SFGSGFGGGY, SEQ ID NO. 2 mimics N-acetyl-beta-D- glucosamine in reaction with antibodies and lectins, and induces in vivo anti-carbohydrate antibody response. *J.Immunol.* 1994;153:5593-5606). Table 2 summarizes the data obtained in that study. This experiment is performed to repeat these studies and to determine the functional activity of these peptides in relationship to MBL activity, binding and complement activation. As seen in Table 2, specific AA substitutions within the GLUPEP backbone either increases, decreases or does not alter the binding of a monoclonal antibody against GlcNAc. Specifically, introduction of lysine (K) at position 1, 2, 6 or 10 significantly increases antibody binding. Similarly, WGA, a lectin with specificity similar to GlcNAc, binding was increased or decreased with the same amino acid substitutions as outlined in Table 2. Aromatic substitution in position 2 with hydrophobic nonaromatic alanine significantly decreased binding. These peptides are

synthesized (>95% pure) and tested in the functional assays disclosed herein. First, the peptides are screened for their ability to inhibit MBL-dependent complement activation in the keratin solid phase ELISA. IC50's are obtained for each peptide. Second, the peptides are coupled to BSA and their ability to activate complement via the lectin pathway using purified complement components (e.g., MBL, C2, C3 and C4) and/or HS is evaluated. Preliminary data using GLUPEP coupled to BSA demonstrated specific MBL binding that was inhibited by 3F8. Specific interactions of selected peptides with MBL are observed by surface plasmon resonance (Biacore). Selected peptides also are evaluated for their ability to inhibit complement activation following oxidative stress of HUVECs. The peptide interaction with UEA-II also is evaluated.

Table 2. Reaction of an anti-GlcNAc mAb (clone CKB-1) to GLUPEP and its substituted variants (adapted from[48]). Similar binding observations were observed with the legume lectin WGA (i.e., a lectin inhibited by GlcNAc).

Peptide	Amino Acid Sequence 1.....10	%Binding
GLUPEP	SFGSGFGGGY, SEQ ID No. 2	100
1	**T**FGSGFGGGY, SEQ ID No. 6	81
2	**A**FGSGFGGGY, SEQ ID No. 7	77
3	**D**FGSGFGGGY, SEQ ID No. 8	28
4	**K**FGSGFGGGY, SEQ ID No. 9	222
5	**SY**GSGFGGGY, SEQ ID No. 10	100
6	**SA**GSGFGGGY, SEQ ID No. 11	25
7	**SD**GSGFGGGY, SEQ ID No. 12	7
8	**SK**GSGFGGGY, SEQ ID No. 13	213
9	SFGSG**K**GGGY, SEQ ID No. 14	175
10	SFGSGFGGG**F**, SEQ ID No. 15	105
11	SFGSGFGGG**A**, SEQ ID No. 16	58
12	SFGSGFGGG**D**, SEQ ID No. 17	16
14	SFGSGFGGG**K**, SEQ ID No. 18	347

Specific substitutions are in **BOLD**.

These experiments establish the structure/activity relationship of these peptides and MBL inhibition. The assays described herein are used to determine which of these peptides display "GlcNAc mimicry" and functionally inhibit MBL. These data then are used to evaluate the energy minimization of these AA conformations. These data are then used to develop small molecular weight inhibitors based on total organic synthesis. IC50 values are obtained for each peptide in each assay.

Example 10. Determination of other peptides that bind to the CRD region of MBL

Historically, MBL is known to only bind to carbohydrate groups. We have made the novel observation that MBL binds to a peptide (GLUPEP). Binding of MBL to GLUPEP in a solid phase ELISA was inhibited by excess GLUPEP, 3F8 or GlcNAc in the fluid phase (Figure 3). These data demonstrate that peptides bind to the CRD of MBL. Thus, it is possible that other families of peptides may bind to the CRD region of MBL and inhibit its function/binding. Identification of these peptide families will aid in the development of small molecular weight inhibitors derived by total organic synthesis.

Other peptides binding to the CRD of MBL are mapped by using commercially available phage display peptide libraries (New England Biolabs). Three separate phage display peptide libraries (New England Biolabs) displaying linear 7-mer ($2x10^9$ independent clones), 7-mer disulfide constrained ($3.7x10^9$ independent clones) and linear 12-mer ($1.9x10^9$ independent clones) are used according to the manufacturers instructions. These two 7-mer libraries are sufficiently complex to contain most if not all of the 20^7 possible 7-mer sequences. The phage are plated and two plaque lifts are performed. The membranes are blocked and purified, functionally, active human MBL are incubated with the membranes. The membranes are washed in calcium and magnesium sufficient buffer and then incubated with our non-functional anti-human MBL mAb, 1C10, conjugated with HRP to identify MBL-positive plaques (ECL system). The second plaque lift is screened with human MBL that is functionally blocked with our other functionally inhibitory mAb to human MBL (clone 3F8). Alternatively, GLUPEP is used to block the MBL CRD. Comparison of the first screen with the second screen eliminates those phage that bind to regions of MBL other than to the CRD. The positive colonies are picked, amplified and sequenced. The corresponding peptides encoded by these phage are synthesized and tested in our screening assays for functional activity against MBL and binding to MBL. Binding of these peptides with MBL is confirmed with surface plasmon resonance (Biacore).

Alternatively, another approach is used if MBL binds to the bacteria used in this system. 1C10 is covalently coupled to protein A Sepharose with a commercially available kit (Pierce). This approach allows specific orientation of the mAb to allow for efficient coupling of functionally active MBL to 1C10. Coupling of MBL in this fashion allows MBL's CRD to be available for direct binding of the phage display peptide libraries. Previous work by

Drs. Stahl and Klickstein have demonstrated by Biacore that the Kd of 1C10 is very low. Saturating concentrations of 3F8 are then added to block the MBL's CRD and the "column" washed. The phage are biopanned across the Sepharose-1C10-MBL-3F8 "column". Again, GLUPEP can be used to block the CRD instead of 3F8. This may be a reasonable approach, as GLUPEP inhibits MBL (above-described results). Those phage that don't bind to the "column" are passed over another Sepharose-1C10-MBL "column" (i.e., no 3F8). The phage that bind to the MBL CRD (i.e., absence of 3F8 → functional CRD on MBL) are eluted with 100 mmol/L GlcNAc, amplified and biopanned 3-4 more rounds to select those peptides with increased specificity of binding. Colonies then are picked and sequenced.

Example 11 Development of functionally inhibitory Fabs.

We have strong data demonstrating that MBL binds to a specific AA sequence conserved in K. Development of functionally inhibitory Fabs (or recombinant scFV) is another therapeutic strategy for the functional inhibition of MBL binding to this peptide sequence. Based on the above-described experiments, additional Fabs to these peptide sequences, relevant to known inflammatory proteins, also are made.

We have coupled GLUPEP to BSA and KLH. Mice are immunized with purified K and/or KLH-GLUPEP and hybridomas are made as we have described (Tofukuji M, Stahl GL, Agah A, Metais C, Simons M, Sellke FW: Anti-C5a monoclonal antibody reduces cardioplegia-induced coronary endothelia dysfunction. *J.Thorac.Cardiovasc.Surg.* 1998;116:1060-1068). The supernatants of GLUPEP hybridomas are screened against BSA-GLUPEP to isolate only those clones that recognize GLUPEP. Monoclonal antibodies from IgG containing parent lines are obtained by limiting dilution. Fabs are made for GLUPEP mAbs. Fabs are used in complement inhibition assays, as classical complement pathway activation would be expected with the use of whole antibodies. Fabs and/or whole antibodies are assayed for their ability to 1) bind to keratin (i.e., K14/17), 2) purification of K14/17 by affinity chromatography from gamma-interferon stimulate human HeLa cells (ATCC) or T84 cells (Dr. Colgan), 3) immunoprecipitation of K from HUVECs following oxidative stress, 4) inhibition (Fabs only) of MBL deposition to keratin and/or HUVECs following oxidative stress, and 5) confocal microscopy of keratin expression on HUVECs.

Kinetics, specificity and affinity of the Fabs are obtained against GLUPEP, K. The ability of the anti-K mAbs to block MBL binding to K (Biacore and solid-phase ELISA) is used as a screening tool to identify functionally inhibitory antibodies.

Once functionally inhibitory antibodies to GLUPEP (e.g., Fabs) are prepared and characterized, humanized scFv's are prepared for potential clinical use. (See, e.g., USSN 60/112,390, entitled "Anti-Mannose (Mannan) Binding Lectin Therapy for Ischemia/Reperfusion Injury" for a description of the methods useful for such purposes.). Since K17 is known to play an important role in psoriasis, allergic reactions and other inflammatory conditions that also involve complement activation, development of specific inhibitors to cytokeratin should have broad utilization in many human diseases.

Example 12. Complement activation during gastrointestinal ischemia and reperfusion is responsible for upregulation of the neutrophil adherence molecule ICAM-1. Adherence of PMN within the gastrointestinal vasculature is dependent on MBL deposition and C5 cleavage, but not C3 degradation products.

We have previously demonstrated *in vivo* that inhibition of complement at the level of C5 (inhibition of C5a and C5b-9 formation) decreases infarct size, apoptosis and PMN accumulation (Vakeva A, Agah A, Rollins SA, Matis LA, Li L, Stahl GL: Myocardial infarction and apoptosis after myocardial ischemia and reperfusion. Role of the terminal complement components and inhibition by anti-C5 therapy. *Circulation* 1998;97:2259-2267). A recent study demonstrated that the C1 esterase inhibitor prevented the upregulation of P-selectin and ICAM-1 in the ischemic/reperfused rat heart *in vivo* (Buerke M, Prüfer D, Dahm M, Oelert H, Meyer J, Darius H: Blocking of classical complement pathway inhibits endothelial adhesion molecule expression and preserves ischemic myocardium from reperfusion injury. *Journal of Pharmacology and Experimental Therapeutics* 1998;286:429-438). However, these authors recognized that the C1 esterase inhibitor also inhibits the MBL pathway and conclusive data on the role of MBL in their study could not be made (Buerke M, Prüfer D, Dahm M, Oelert H, Meyer J, Darius H: Blocking of classical complement pathway inhibits endothelial adhesion molecule expression and preserves ischemic myocardium from reperfusion injury. *Journal of Pharmacology and Experimental Therapeutics* 1998;286:429-438). Others have reported that C5b-9 directly induces ICAM-1, MCP-1, ELAM and IL-8 in HUVECs (Kilgore KS, Flory CM, Miller BF, Evans VM, Warren JS: The membrane attack

complex of complement induces interleukin-8 and monocyte chemoattractant protein-1 secretion from human umbilical vein endothelial cells. *Am.J.Pathol.* 1996;149:953-961; Kilgore KS, Schmid E, Shanley TP, Flory CM, Maheswari V, Tramontini NL, Cohen H, Ward PA, Friedl HP, Warren JS: Sublytic concentrations of the membrane attack complex of complement induce endothelial interleukin-8 and monocyte chemoattractant protein-1 through nuclear factor-kappaB activation. *Am.J.Pathol.* 1997;150:2019-2031; Kilgore KS, Shen JP, Miller BF, Ward PA, Warren JS: Enhancement by the complement membrane attack complex of tumor necrosis factor-α-induced endothelial cell expression of E-selectin and ICAM-1. *J.Immunol.* 1995;155:1434-1441). C5b-9 and C5a also directly upregulate CD62P on vascular endothelial cells (Mulligan MS, Schmid E, Till GO, Hugli TE, Friedl HP, Roth RA, Ward PA: C5a-dependent up-regulation in vivo of lung vascular P-selectin. *J.Immunol.* 1997;158:1857-1861; Hattori R, Hamilton KK, McEver RP, Sims PJ: Complement proteins C5b-9 induce secretion of high molecular weight multimers of endothelial von Willebrand factor and translocation of granule membrane protein GMP-140 to the cell surface. *J.Biol.Chem.* 1989;264:9053-9060). Complement activation by immune complexes induces two rat chemokines, macrophage inflammatory protein-2 (MIP-2) and cytokine-induced neutrophil chemoattractant (CINC) (Shanley TP, Schmal H, Warner RL, Schmid E, Friedl HP, Ward PA: Requirement for C-X-C chemokines (Macrophage inflammatory protein-2 and cytokine-induced neutrophil chemoattractant) in IgG immune complex-induced lung injury. *J.Immunol.* 1997;158:3439-3448). PMN sequestration in the isolated ischemic/reperfused rat heart is dependent on complement and is inhibited by anti-CD18 or sCR1 (Lefer DJ, Shandelya SML, Serrano CV, Jr., Becker LC, Kuppusamy P, Zweier JL: Cardioprotective actions of a monoclonal antibody against CD-18 in myocardial ischemia-reperfusion injury. *Circulation* 1993;88:1779-1787). We have demonstrated recently that C5b-9 induces VCAM-1 protein expression by decreasing intracellular cGMP and inducing nuclear translocation of NFκB. The experiments presented below are intended to investigate the biochemical and molecular aspects of UEA-II treatment and C5 inhibition on PMN adherence and the induction of ICAM-1 in the ischemic/reperfused rat GI tract.

The SMA of rats are occluded for 1 hr and reperfused (2-6 hrs). The rats are separated into the following groups: 1) sham; 2) vehicle control; 3) anti-C5 mAb (18A; 20 mg/kg); 4) UEA-II (0.01 – 1 mg/kg); and 5) GLUPEP mimetic (dose and structure to be determined by the in vitro findings outlined above). (16C is used as the isotype and

nonfunctional control for 18A as we have described (Vakeva A, Agah A, Rollins SA, Matis LA, Li L, Stahl GL: Myocardial infarction and apoptosis after myocardial ischemia and reperfusion. Role of the terminal complement components and inhibition by anti-C5 therapy. *Circulation* 1998;97:2259-2267). Scrambled peptides are used as controls for the GLUPEP mimetic.)

At the end of the reperfusion period, the area at risk (blue dye negative) is removed and assessed for ICAM-1 mRNA expression by semi-quantitative RT-PCR. GAPDH or β-actin is used as a housekeeping gene and control for mRNA loading. These results are confirmed by northern analysis and *in situ* hybridization coupled to confocal microscopy as we described (Collard CD, Bukusoglu C, Agah A, Colgan SP, Reenstra WR, Morgan BP, Stahl GL: Hypoxia-induced expression of complement receptor type 1 (CR1, CD35) in human vascular endothelial cells. *Am.J.Physiol.* 1999;276:C450-C458). Commercially available primer sets are used or designed from published sequences. Immunohistochemical staining for CD62P and ICAM-1 will be done as previously described (Buerke M, Prüfer D, Dahm M, Oelert H, Meyer J, Darius H: Blocking of classical complement pathway inhibits endothelial adhesion molecule expression and preserves ischemic myocardium from reperfusion injury. *Journal of Pharmacology and Experimental Therapeutics* 1998;286:429-438) . MPO activity is assessed as an index of PMN infiltration as described (Vakeva A, Agah A, Rollins SA, Matis LA, Li L, Stahl GL: Myocardial infarction and apoptosis after myocardial ischemia and reperfusion. Role of the terminal complement components and inhibition by anti-C5 therapy. *Circulation* 1998;97:2259-2267).

We expect to observe complement activation (C5b-9 deposition) and PMN adherence within the gastrointestinal tract in vehicle treated animals, but not in UEA-II or the anti-C5 antibody (i.e., 18A) treated animals. We also expect to observe significant increases in ICAM-1 mRNA expression in the splanchnic region of vehicle treated rats and decreased expression within UEA-II or anti-C5 treated animals. These data support our previous findings for a role of C5 cleavage products in the upregulation of PMN adherence molecules and extend these findings to show the effectiveness of UEA-II *in vivo* (Vakeva A, Agah A, Rollins SA, Matis LA, Li L, Stahl GL: Myocardial infarction and apoptosis after myocardial ischemia and reperfusion. Role of the terminal complement components and inhibition by anti-C5 therapy. *Circulation* 1998;97:2259-2267). We expect to observe decreased C3 deposition in UEA-II treated animals, but do not expect iC3b to play a major role as a PMN

ligand as we demonstrated previously in the myocardium (Vakeva A, Agah A, Rollins SA, Matis LA, Li L, Stahl GL: Myocardial infarction and apoptosis after myocardial ischemia and reperfusion. Role of the terminal complement components and inhibition by anti-C5 therapy. *Circulation* 1998;97:2259-2267). Experiments using the anti-C5 antibody and UEA-II demonstrate that MBL activates complement and the terminal C5 molecule is necessary for adherence molecule expression in the ischemic/reperfused gut. It is possible that the production of reactive oxygen species in these experiments increases adherence molecule and cytokine expression (Gasic AC, McGuire G, Krater S, Farhood AI, Goldstein MA, Smith CW, Entman ML, Taylor AA: Hydrogen peroxide pretreatment of perfused canine vessels induces ICAM-1 and CD18-dependent neutrophil adherence. *Circulation* 1991;84:2154-2166). Indeed, we have demonstrated that hypoxia enhances cytokine mediated ICAM expression in human endothelial cells (Zund G, Uezono S, Stahl GL, Dzus AL, McGowan FX, Hickey PR, Colgan SP: Hypoxia enhances induction of endothelial ICAM-1: role for metabolic acidosis and proteasome activation. *Am.J.Physiol.* 1997;273:C1571-C1580). We believe that the complement system however, will amplify this response and increase the overall inflammatory condition. This finding is consistent with our previous *in vitro* findings (Zund G, Uezono S, Stahl GL, Dzus AL, McGowan FX, Hickey PR, Colgan SP: Hypoxia enhances induction of endothelial ICAM-1: role for metabolic acidosis and proteasome activation. *Am.J.Physiol.* 1997;273:C1571-C1580). Thus, inhibition of complement at C5 or UEA-II treatment attenuates complement-mediated increases in the inflammatory process. CD47 is involved in PMN trafficking in the gastrointestinal system (Parkos CA, Colgan SP, Liang TW, Nusrat A, Bacarra AE, Carnes DK, Madara JL: CD47 mediates post-adhesive events required for neutrophil migration across polarized intestinal epithelia. *J.Cell Biol.* 1996;132:437-450). We have also shown that C5b-9 induces CD47 expression in epithelial cells. We also investigate the effect of anti-C5 and UEA-II treatment on this important PMN adherence molecule. At least 6-10 rats are used in each group to establish a mean and SEM. Appropriate statistical analysis is done.

Through different experimental techniques, we are able to establish the affinities of GLUPEP (this is possible for other conjugates and/or amino acid sequences related to GLUPEP too). Further, with this new experimental application (i.e., GlcNAc-BSA and BIAcore) we are able to ligand fish either chemical, phage display and other libraries for those molecules that either bind to GlcNAc-BSA and inhibit MBL binding or bind to MBL

and inhibit MBL binding to the GlcNAc-BSA chip. This new technique will allow us to rapidly screen hybridomas that we make to keratin or other MBL ligands to identify those antibodies that specifically bind to the ligand and inhibit MBL binding. As shown in Figure 6, GLUPEP inhibited MBL binding to GlcNAc-BSA in a concentration related manner. Specific affinities of these novel inhibitors will be calculated using the BIAcore 3000. (GLUPEP inhibited native MBL (10μg/ml) binding in a concentration dependent manner, The beginning and ending of the MBL injection on the chip is denoted in the figure with arrows. Time is in seconds.)

Example 13. UEA-II Significantly Decreases Oxidative Stress Induced Neutrophil Chemotaxis in Endothelial Cells.

As previously demonstrated, UEA-II recognizes a MBL ligand on human endothelial cells following oxidative stress. By selectively blocking MBL binding to its ligand, UEA-II inhibits complement activation in HUVEC cells. The activation of the complement pathway on endothelial cells leads to the generation of potent chemoattractants including the anaphylatoxin C5a, IL-8, and monocyte chemoattractant protein (Kilgore, K.S., et al., *Am. J. Pathol.* 149, 953-961 (1996), Saadi, S., *Circulation* 101, 1867-1873)). Neutrophil chemotaxis following oxidative stress was therefore utilized as an assay of UEA-II activity in HUVEC cells. We hypothesized that UEA-11 would decrease complement-mediated neutrophil chemotaxis following endothelial oxidative stress.

Reoxygenation of hypoxic HUVEC in HS significantly ($p < 0.05$) increased neutrophil chemotaxis compared to normoxic cells bathed in HS. As predicted, treatment with UEA-II (100 nmol/L). significantly attenuated neutrophil chemotaxis following endothelial oxidative stress compared to vehicle-treated cells. Neutrophil chemotaxis to HUVEC following oxidative stress was measured by analysis of myeloperoxidase levels and transformed to a neutrophil count with a standard curve using known numbers of neutrophils. This experiment was performed 3 times, with 3 wells per experimental group (n=3).

Treatment of normoxic cells with UEA-II had no effect on fMLP driven chemotaxis compared to untreated normoxic HUVEC (data not shown), which demonstrates that the effect of UEA-II on chemotaxis is not a non-specific effect on PMN function. These data demonstrate that in endothelial cells UEA-II attenuates complement-mediated neutrophil chemotaxis induced by oxidative stress.

Methods

HUVEC Studies

HUVEC isolation. HUVECs will be isolated, cultured and purity established as we previously demonstrated from human umbilical veins (Collard CD, Vakeva A, Bukusoglu C, Zund G, Sperati CJ, Colgan SP, Stahl GL: Reoxygenation of hypoxic human umbilical vein endothelial cells (HUVECs) activates the classic complement pathway. *Circulation* 1997;96:326-333; Collard CD, Agah A, Stahl GL: Complement activation following reoxygenation of hypoxic human endothelial cells: role of intracellular reactive oxygen species, NF- kappaB and new protein synthesis. *Immunopharmacology* 1998;39:39-50; Zund G, Uezono S, Stahl GL, Dzus AL, McGowan FX, Hickey PR, Colgan SP: Hypoxia enhances induction of endothelial ICAM-1: role for metabolic acidosis and proteasome activation. *Am.J.Physiol.* 1997;273:C1571-C1580) . Cells will be used during passage 1-3. Cells will be grown in flasks, petri dishes and 96-well plates as required for the experiments outlined in this proposal.

Hypoxia induction. Hypoxia will be induced as we have previously demonstrated (Collard CD, Vakeva A, Bukusoglu C, Zund G, Sperati CJ, Colgan SP, Stahl GL: Reoxygenation of hypoxic human umbilical vein endothelial cells (HUVECs) activates the classic complement pathway. *Circulation* 1997;96:326-333; Collard CD, Agah A, Stahl GL: Complement activation following reoxygenation of hypoxic human endothelial cells: role of intracellular reactive oxygen species, NF- kappaB and new protein synthesis. *Immunopharmacology* 1998;39:39-50; Zund G, Uezono S, Stahl GL, Dzus AL, McGowan FX, Hickey PR, Colgan SP: Hypoxia enhances induction of endothelial ICAM-1: role for metabolic acidosis and proteasome activation. *Am.J.Physiol.* 1997;273:C1571-C1580) . Briefly, the cells are placed in a commercially available, microprocessor controlled, sealed, humidified, temperature controlled and gloved chamber (Coy). Oxygen concentration is regulated to 1% O_2 and maintained for the experimental period. Cells are placed in the chamber for the specified period of time, removed and assayed as described in the Research Plan outlined above. This amount of oxygen decreases media PO_2 to 14-16 mm Hg within 8 hr (unpublished observations). Our previous findings demonstrate that complement activation during hypoxia is augmented by reoxygenation in a time-dependent manner (Collard CD, Vakeva A, Bukusoglu C, Zund G, Sperati CJ, Colgan SP, Stahl GL:

Reoxygenation of hypoxic human umbilical vein endothelial cells (HUVECs) activates the classic complement pathway. *Circulation* 1997;96:326-333; Collard CD, Agah A, Stahl GL: Complement activation following reoxygenation of hypoxic human endothelial cells: role of intracellular reactive oxygen species, NF- kappaB and new protein synthesis. *Immunopharmacology* 1998;39:39-50) .

Cell surface biotinylation. Cell surface biotinylation of proteins will be done as we previously described (Collard CD, Vakeva A, Bukusoglu C, Zund G, Sperati CJ, Colgan SP, Stahl GL: Reoxygenation of hypoxic human umbilical vein endothelial cells (HUVECs) activates the classic complement pathway. *Circulation* 1997;96:326-333).

MBL ELISA. Turner and colleagues have previously described the MBL ELISA (*Super M, Levinsky RJ, Turner MW: The level of mannan-binding protein regulates the binding of complement- derived opsonins to mannan and zymosan at low serum concentrations.* Clin.Exp.Immunol. *1990;79:144-150*). Briefly, 96-well microtiter plates are coated with mannan (500 μg/ml; 50 μl each well). Human sera (1-4%) with or without GlcNAc (30 mmol/L) is added to the wells and incubated for 30 min at 37°C. The plates are then washed and developed with a polyclonal antibody to human C3 (Cappel) as previously described *(Collard CD, Vakeva A, Bukusoglu C, Zund G, Sperati CJ, Colgan SP, Stahl GL: Reoxygenation of hypoxic human umbilical vein endothelial cells (HUVECs) activates the classic complement pathway.* Circulation *1997;96:326-333).* MBL-dependent C3 deposition to plastic is inhibited by GlcNAc or our functionally inhibitory mAbs against MBL in this assay. We will use this assay to screen functionally inhibitory peptides, to evaluate pharmacokinetic and pharmacodynamic properties of the peptides, and to assess therapeutic doses of MBL inhibitors ex vivo.

Keratin ELISA. We have developed this assay along the same lines as the MBL ELISA. Briefly, 96-well microtiter plates are coated with keratin (2 μg/ml; 50 μl each well). Human sera (4-30%) with or without GlcNAc (30 mmol/L) or 3F8 (2 μg/ml) is added to the wells and incubated for 30 min at 37°C. The plates are then washed and developed with a polyclonal antibody to human C3 (Cappel) as previously described *(Collard CD, Vakeva A, Bukusoglu C, Zund G, Sperati CJ, Colgan SP, Stahl GL: Reoxygenation of hypoxic human umbilical vein endothelial cells (HUVECs) activates the classic complement pathway.* Circulation *1997;96:326-333).* MBL-dependent C3 deposition to plastic is inhibited by GlcNAc or 3F8 (our functionally inhibitory mAb against MBL) in this assay. We will use

this assay to screen functionally inhibitory peptides and Fabs, to evaluate pharmacokinetic and pharmacodynamic properties of the peptides, and to assess therapeutic doses of MBL inhibitors *ex vivo*.

Antibody production. Antigenic material is purified and emulsified with Titer Max. (NOTE: We have unpublished observations that the immune response to Titer Max is greater than Freund's adjuvant, giving higher antibody titers in a shorter time span and a predominance of IgG subclasses and virtually no IgM.) The mice or rabbits are immunized first with the Titer Max suspension and boosted weekly for 3-4 weeks to produce the immune response. Rabbits are then bled and the polyclonal antibodies purified by protein G purification. Mice spleens are removed and fused to produce hybridomas as previously described (Tofukuji M, Stahl GL, Agah A, Metais C, Simons M, Sellke FW: Anti-C5a monoclonal antibody reduces cardioplegia-induced coronary endothelia dysfunction. *J.Thorac.Cardiovasc.Surg.* 1998;116:1060-1068). Hybridomas are then screened for those producing only IgG isotypes and recognizing the antigen. The secondary screen is a functional screen and those clones of interest are limited diluted to produce a monoclonal cell line. Antibodies are isotyped (Gibco) and purified from tissue culture supernatant by Protein A/G affinity chromatography.

Neutrophil Chemotaxis Assay. HUVEC were grown to confluence on 24-well plates and then subjected to o or 24 hour of hypoxia. Following the specified period of normoxia or hypoxia, the media was aspirated and the cells reoxygenated (3 hours) in the presence of 30% HS or 30% HS treated with UEA-II (100 nmol/L). During the reoxygenation period, human neutrophils were harvested and isolated as previously described (Henson, P.M. et al., *J. Clin. Invest.* 56, 1053-1061 (1975)). Five-micron transwell inserts (Corning Costar, Cambridge, MA) were then placed in each well of the reoxygenated HUVEC. Human neutrophils (2 x 10^6 cells/well) were added to each transwell and incubated for 90 min at 37° C. The supernatant covering the HUVEC was removed and centrifuged at 150 x g for 10 minutes. The resulting pellet was resuspended in 1 ml of HBSS, solubilized with 50 µl of 10% Triton x -100 and acidified with 100 µl of citrate buffer (1 mol/L pH 6.5). The myeloperoxidase (MPO) content of the wells was then assayed as previously described (Parkos, C.A., et al., *J. Cell Biol.* 117, 757-764 (1992)).

Molecular Biology

RT-PCR. Rat tissue will be removed and frozen in liquid nitrogen. Total RNA from homogenized rat tissue or HUVECs is extracted using a commercially available product containing guanidinium isothiocyanate/chloroform (TRIzol, Life Technologies). The poly A mRNA is purified by oligo(dT) cellulose (Promega). First strand cDNA synthesis is constructed using reverse transcription, 1 μg poly A mRNA and a commercially available kit (Promega). cDNA amplification of rat ICAM-1 will be done using primers sets as described (Beck-Schimmer B, Schimmer RC, Schmal H, Flory CM, Friedl HP, Pasch T, Ward PA: Characterization of rat lung ICAM-1. *Inflamm.Res.* 1998;47:308-315; Lee SK, Park JY, Chung SJ, Yang WS, Kim SB, Park SK, Park JS: Chemokines, osteopontin, ICAM-1 gene expression in cultured rat mesangial cells. *J.Korean.Med.Sci.* 1998;13:165-170) . Rat β-actin or GAPDH (housekeeping genes) will be used to control for RNA loading conditions and run in the gels with the other PCR products. Northern analysis and *in situ* hybridization for will be used to validate the PCR findings as we have described (Collard CD, Bukusoglu C, Agah A, Colgan SP, Reenstra WR, Morgan BP, Stahl GL: Hypoxia-induced expression of complement receptor type 1 (CR1, CD35) in human vascular endothelial cells. *Am.J.Physiol.* 1999;276:C450-C458). Controls for RT-PCR will include PCR in the absence of RT. Primer sets for human keratin are listed below.

Human keratin 14: Forward: TTCTGAACGAGATGCGTGAC, SEQ ID NO. 19
(product size: 448 bp)
Reverse: AGAACTGGGAGGAGGAGAG, SEQ ID NO. 20

Human keratin 17: Forward: ATTGGCAGCGTGGGAGGA, SEQ ID NO. 21
(product size: 332 BP)
Reverse: AGACTGTGGGGCAGATGG, SEQ ID NO. 22

Peptide phage display library. To map potential binding sites of peptides to MBL, we will use phage display peptide libraries from New England Biolabs (NEB) according to their instructions. NEB and others have used these libraries to identify consensus peptide binding sequences against streptavidin, monoclonal and polyclonal antibodies, RNase A, PAP kinase and cell-surface receptors (communication from NEB). Specific methods are given by the manufacture and will be modified as we have described in the Research Design

Section 3b. It takes a minimum of 4-6 weeks for a single round of biopanning and sequencing for each screening.

MBL purification. We have successfully purified human, rat and porcine MBL using a modification of published procedures (Tan SM, Chung MCM, Kon OL, Thiel S, Lee SH, Lu J: Improvements on the purification of mannan-binding lectin and demonstration of its Ca^{2+}-independent association with a C1s- like serine protease. *Biochem.J.* 1996;319:329-332). This method isolates MBL associated with MASP1 and MASP2. Briefly, plasma is precipitated with PEG3500 (10%; w:v). The pellet is then dissolved in a calcium buffer and applied to a mannan affinity column (Sigma). Bound material is eluted from the mannan column with EDTA containing buffer. Protein positive tubes are collected and re-calcified and applied to a small maltose column. MBL is eluted from the maltose column with GlcNAc (100 mmol/L). The MBL is dialyzed against PBS containing 0.5 mmol/L NaCl, sterile filtered and stored at 4°C. Western analysis of this material demonstrates MBL and the lack of contaminating IgG or IgM.

Isolation and purification of human CK1. Human CK1 was purified from human dermal keratin (Sigma, St. Louis, MO) using a monospecific rabbit anti-human CK1 polyclonal antibody (pAb) (Convance/BAbCO, Richmond, CA) conjugated to protein G Sepharose (ImmunoPure Protein G IgG Plus orientation Kit; Pierce, Rockford IL). After equilibrating the protein G column with binding buffer (10 mmol/L TRIS, pH 7.5), human keratin was loaded onto the column. The column was then washed extensively with binding buffer and eluted with 0.1 mol/L glycine-HCl buffer, pH 2.8, with the eluent being collected in 0.5 ml fractions containing 1 mol/L TRIS-HCl buffer (1:10; v:v), pH 9.5. The protein-containing fractions were pooled, dialyzed overnight in 10 mmol/L TRIS buffer, pH 7.5, and the protein concentration determined.

Generation of anti-human keratin antibodies and Fab fragments. Male NZW rabbits (Harlan, Indianapolis, IN) were immunized initially with human keratin (100 μg, s.c.) in TiterMax (Sigma, St. Louis, MO) and then with human keratin (50 μg, s.c.) in PBS on a biweekly basis for 6 weeks. Two weeks after the last immunization, the animals were bled and the resultant pAb purified by protein G affinity chromatography. All pAb were dialyzed against PBS, concentrated and sterile filtered.

Polyclonal anti-human keratin Fab fragments were generated by digesting anti-human keratin pAb with papain (Sigma, St. Louis, MO) for 16 hr at 37 °C. The reaction was

terminated with iodoacetamide (Sigma, St. Louis, MO). The resulting mixture was then dialyzed in PBS, pH 8.0 overnight at 4 °C. Any remaining whole IgG and the Fc portion of the anti-human keratin pAb were removed from the mixture by protein A affinity chromatography. Fab fragment generation was confirmed by SDS-PAGE.

HUVEC CK1 ELISA. Confluent HUVEC were subjected to 0 or 24 hr of hypoxia (1% O_2). The cell media were aspirated and 100 µl of gelatin-veronal buffer (GVB) containing Ca^{2+}/Mg^{2+} added to each well. The cells were then reoxygenated for 3 hr at 37 °C, washed and fixed with 1% paraformaldehyde (Sigma, St. Louis, MO) for 30 min. After washing, the cells were incubated with 50 µl of rabbit anti-human CK1 pAb (1:500 dilution; Convance/BAbCO, Richmond, CA) or anti-porcine C7 pAb (20 µg/ml; isotype control) for 1 hr at 4 °C. After washing, 50 µl of peroxidase-conjugated goat anti-rabbit pAb was added to each well and incubated for 1 hr at 4 °C. The plates were washed, developed with 50 µl of ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid] and read (Molecular Devices, Sunnyvale CA) at 405 nm. Background optical density was determined from cells to which only the secondary antibody was added, and was subtracted from all groups. This experiment was performed 3 times using 3 wells per experimental group (n=3).

Immunoprecipitation and Sequencing of HUVEC CK1. To confirm the specificity of the anti-human CK1 pAb used in these experiments, HUVEC CK1 was immunoprecipitated and sequenced. Confluent HUVEC cultures grown in 100 mm Petri dishes were subjected to 24 hr of hypoxia followed by 3 hr of reoxygenation in the presence of GVB. The cells were then washed with ice cold GVB and incubated with lysing buffer (150 mmol/L NaCl, 25 mmol/L Tris, 1 mmol/L $MgCL_2$, 1% Triton X-100, 1% Nonidet P-40, 5 mmol/L EDTA, 5 µg/ml chymostatin, 2 µg/ml aprotinin, and 1.25 mmol/L PMSF, pH 7.4, all from Sigma Chemicals). Cell debris was removed by centrifugation (10,000 x g; 5 min). Cell lysates were pre-cleared with 50 µl of pre-equilibrated protein-G Sepharose (Pharmacia, Uppsala, Sweden) overnight at 4 °C. CK1 immunoprecipitation was performed by addition of rabbit anti-human CK1 pAb (4 µg/ml; Convance/BAbCO, Richmond, CA). Following centrifugation (10,000 x g; 5 min) and washing, the immunoprecipitates were boiled in reducing sample buffer and separated by SDS-PAGE. After staining with Coomassie blue, the resultant protein band was cut from the gel and sent to the Harvard University Core Microchemistry Facility for microsequencing.

C3 and MBL Deposition (ELISA) on Purified CK1. Purified human CK1 (50 µl; 2 µg/ml in 15 mmol/L sodium carbonate, pH 9.6) was added to 96-well microtiter plates for 12-16 hr at 4 °C. After washing, the plates were blocked for 2 hr at room temperature with 3% bovine serum albumin (BSA) and washed again. HS (2% final concentration) was incubated with a) 100 mmol/L GlcNAc; b) 20 µg/ml anti-human MBL mAb, 3F8 (Collard et al., 2000); or c) vehicle [veronal buffered saline (VBS) containing Ca^{2+}/Mg^{2+}] for 30 min at room temperature. The plates were then inoculated with 100 µl of treated or untreated HS, and incubated for 30 min at 37 °C. The plates were washed and 50 µl of HRP-conjugated goat anti-human C3 pAb (1:2000 dilution; ICN, Aurora, OH) or rabbit anti-human MBL pAb (R2.2; 1:500 dilution) added for 1 hr at room temperature. The plates were then washed and developed as described above. Background optical density was determined from wells coated with BSA only and was subtracted from all groups. This experiment was performed 3-4 times using 3 wells per experimental group (n =3-4).

Immunoprecipitation and Western Blot of human CK1 and MBL. Confluent HUVEC cultures grown on 100 mm Petri dishes were subjected to 0 or 24 hr of hypoxia followed by 3 hr of reoxygenation in the presence of GVB (for CK1 analysis) or 30% HS (for MBL analysis). The cells were then washed with ice cold GVB and incubated with lysing buffer. Cell debris was removed by centrifugation (10,000 x g; 5 min). Cell lysates were pre-cleared with 50 µl of pre-equilibrated protein-G Sepharose. The lysates were then immunoprecipitated by addition of human MBL (90 µg) and 50 µl of anti-human MBL mAb (1C10) (Collard et al., 2000) or anti-human CK1 pAb (Convance/BAbCO, Richmond, CA) conjugated to protein-G Sepharose (ImmunoPure Protein G IgG Plus orientation Kit; Pierce, Rockford IL). Following centrifugation (10,000 x g; 5 min) and washing, the immunoprecipitates were boiled in reducing sample buffer and separated by SDS-PAGE. The gel was electroblotted to nitrocellulose and blocked with 10% non-fat dry milk (NFDM) overnight at 4°C.

For CK1 analysis, anti-human CK1 pAb (1:500 dilution) was incubated with the nitrocellulose in 3% NFDM for 1 hr at 4°C. The nitrocellulose was then washed and incubated with HRP-conjugated goat anti-rabbit pAb (1:1000 dilution; ICN, Aurora, OH) for 1 hr at 4°C. For MBL analysis, HRP-conjugated anti-human MBL mAb (2A9; 1:2000

dilution) was incubated with the nitrocellulose in 3% NFDM for 1 hr at 4°C. The membranes were then washed and developed with the ECL system (Amersham) and x-ray films (Kodak).

HUVEC C3 and MBL ELISA. HUVEC C3 and MBL deposition following oxidative stress was measured by ELISA as previously described (Collard et al., 2000). HUVEC were grown to confluence and then subjected to 0 (normoxia) or 24 hr of hypoxia (1% O_2). The cell media were aspirated and 100 µl of one of the following was added to each well: 1) 30% HS, 2) GVB, 3) 30% HS + 100 mmol/L GlcNAc, 4) 30% HS + 50 µg/ml anti-human keratin pAb or 5) 30% HS + 20 µg/ml anti-human keratin Fab fragments. The cells were then reoxygenated for 3 hr at 37 °C, washed and fixed with 1% paraformaldehyde for 30 min. After washing, the cells were incubated with HRP-conjugated goat anti-human C3 pAb (1:1000 dilution; Cappel, West Chester, PA) or anti-human MBL mAb, 1C10 (1:1000 dilution) for 1 hr at 4 °C. The plates were then washed and developed as described above. Background optical density was determined from cells to which only the anti-human C3 or MBL antibody was added, and was subtracted from all groups. These experiments were performed 3 times using 4-6 wells per experimental group (n=3).

Immunofluorescent confocal microscopy. HUVEC were grown on LabTech tissue culture microscope slides (NUNC) were subjected to 0 or 24 hr of hypoxia and then reoxygenated for 3 hr in 30% HS treated with PBS (vehicle), anti-human keratin Fab fragments (20 µg/ml) or GlcNAc (100 mmol/L). The slides were then washed in PBS containing calcium and magnesium and fixed in 4% paraformaldehyde for 15 min, washed again and blocked with 10% goat serum. Human MBL deposition (green) was identified using biotinylated 1C10 and streptavidin-conjugated FITC (Jackson Immunoresearch, West Grove, PA). Human C3 deposition (green) was evaluated with a FITC-conjugated goat anti-human C3 $F(ab')_2$ antibody (ICN, Aurora, OH). Following incubation with the appropriate antibodies, the slides were washed (x3; 10 min each) and incubated with propidium iodide (10 µg/ml; Sigma). The slides were , then coated with anti-fade mounting media (Molecular Probes, Eugene, OR), covered and analyzed with a Zeiss confocal microscope as previously described (Collard et al., 1999). Controls with streptavidin-conjugated FITC only were processed as above, omitting the primary antibody to determine nonspecific binding. All analyses were conducted at the same pinhole, voltage and laser settings. This experiment was performed three times (n=3).

Statistical Analysis. All data presented represent the mean and SEM for n determinations. Data analyses were performed using Sigma Stat (Jandel Scientific, San Rafael, CA). A p value of <0.05 was considered significant. Endothelial CK1 expression and MBL / C3 deposition on purified CK1-coated plates were analyzed by one-way analysis of variance (ANOVA). Endothelial C3 and MBL deposition on normoxic vs. hypoxic HUVEC were analyzed by two-way ANOVA. All pairwise multiple comparisons were made using the Student-Newman-Keuls test. MBL and C3 deposition on purified CK1-coated plates (ELISA; Fig. 3) were normalized to untreated 2% HS. Means ± SEM of the raw data used for normalization are presented in the results and/or figure legends.

CLAIMS

1. A method for inhibiting LCP associated complement activation, comprising contacting a mammalian cell having surface exposed MBL ligand with an effective amount of an MBL receptor antagonist to inhibit LCP-associated complement activation.

2. The method of claim 1, wherein the MBL receptor antagonist is a legume derived lectin or a functional equivalent thereof that binds to the MBL ligand and that inhibits complement activation.

3. The method of claim 1, wherein the MBL receptor antagonist is an anti- keratin antibody.

4. The method of claim 1, wherein the MBL receptor antagonist is an anti- keratin antibody fragment.

5. The method of claim 2, wherein the MBL receptor antagonist is keratin binding molecule.

6. The method of claim 2, wherein the legume derived lectin is a peptide isolated from a legume derived lectin selected from the group consisting of *Ulex europaeus* (UEA)-II, *Laburnum alpinum* (LAA)-I, and Cytisus Sessilifolius anti-H(O) Lectin 1 (CSA-1).

7. The method of claim 1, wherein the MBL receptor antagonist is an isolated peptide fragment of a legume derived lectin selected from the group consisting of *Ulex europaeus* (UEA)-II, *Laburnum alpinum* (LAA)-I, and Cytisus Sessilifolius anti-H(O) Lectin 1 (CSA-1).

8. The method of claim 2, wherein the method is a screening assay.

9. A method for inhibiting a cellular injury mediated by LCP associated complement activation, comprising administering to a subject in need thereof, an effective amount of an MBL receptor antagonist to inhibit LCP-associated complement activation.

10. The method of claim 9, wherein the MBL receptor antagonist is a legume derived lectin or a functional equivalent thereof that binds to the MBL ligand and that inhibits complement activation.

11. The method of claim 10, wherein the legume derived lectin is a peptide isolated from a legume derived lectin selected from the group consisting of *Ulex europaeus* (UEA)-II, *Laburnum alpinum* (LAA)-I, and Cytisus Sessilifolius anti-H(O) Lectin 1 (CSA-1).

12. The method of claim 10, wherein the MBL receptor antagonist is an isolated peptide selected from the group consisting of the peptide fragments of a legume derived lectin selected from the group consisting of *Ulex europaeus* (UEA)-II, *Laburnum alpinum* (LAA)-I, and Cytisus Sessilifolius anti-H(O) Lectin 1 (CSA-1).

13. The method of claim 6, wherein the MBL receptor antagonist is an anti- keratin antibody.

14. The method of claim 6, wherein the MBL receptor antagonist is an anti- keratin antibody fragment.

15. The method of claim 6, wherein the MBL receptor antagonist is keratin binding molecule.

16. The method of claim 9, wherein the cellular injury mediated by LCP associated complement activation contributes to tissue injury associated with atherosclerosis.

17. The method of claim 9, wherein the cellular injury mediated by LCP associated complement activation contributes to tissue injury associated with the pulmonary system.

18. The method of claim 9, wherein the MBL receptor antagonist is administered to the subject by an aerosol route of delivery or by localized delivery.

19. The method of claim 9, wherein the cellular injury mediated by LCP associated complement activation contributes to tissue injury associated with a disorder selected from the group consisting of arthritis, myocardial infarction, ischemia, reperfusion, transplantation, CPB, stroke, ARDs, SLE, lupus, and dialysis.

20. A composition, comprising an isolated MBL receptor antagonist, and a pharmaceutically acceptable carrier, wherein the MBL receptor antagonist is selected from the group consisting of a legume derived lectin and an isolated peptide fragment of a legume derived lectin.

21. The composition of claim 20, wherein the composition is a pharmaceutical composition including an effective amount for treating an MBL mediated disorder of the isolated MBL receptor antagonist.

22. The composition of claim 21, further comprising a drug for the treatment of an MBL mediated disorder.

23. The composition of claim 20, wherein the isolated peptide fragment of a legume derived lectin is selected from the group consisting of *Ulex europaeus* (UEA)-II, *Laburnum alpinum* (LAA)-I, and Cytisus Sessilifolius anti-H(O) Lectin 1 (CSA-1).

24. The composition of claim 20, wherein the legume derived lectin is a peptide isolated from a legume derived lectin selected from the group consisting of *Ulex europaeus* (UEA)-II, *Laburnum alpinum* (LAA)-I, and Cytisus Sessilifolius anti-H(O) Lectin 1 (CSA-1).

25. A method for screening a subject for susceptibility to treatment with MBL receptor antagonist, comprising:

contacting a mammalian cell from a subject with a labeled isolated MBL receptor antagonist, and detecting the presence of an MBL ligand on the surface of the mammalian

cell, wherein the presence of the MBL ligand indicates that the cell is susceptible to LCP-associated complement activation and that the subject is susceptible to treatment with an MBL receptor antagonist.

26. A screening method for identifying compounds which bind to a human MBL receptor, comprising:

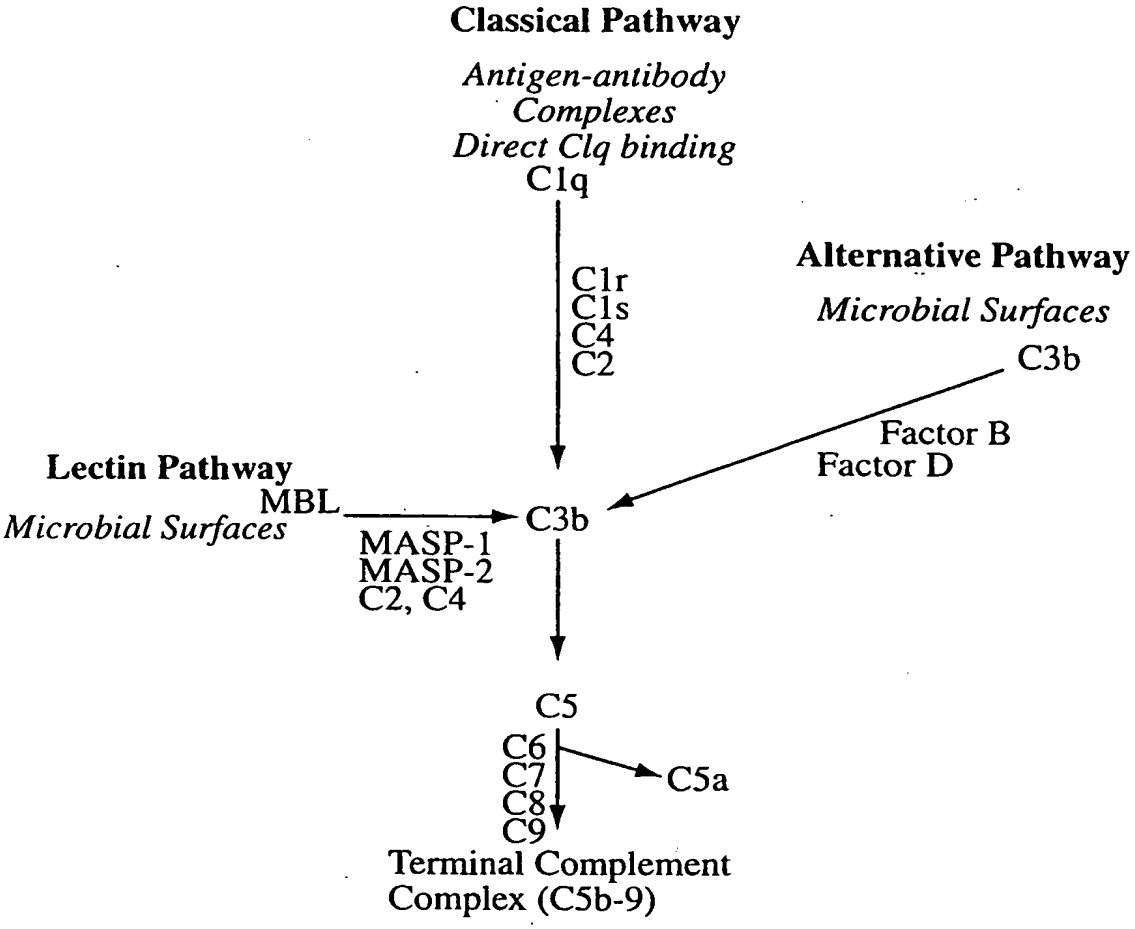
contacting the MBL receptor with a sample containing at least one candidate MBL receptor antagonist,

determining if the at least one candidate MBL receptor antagonist bind to the MBL receptor.

27. The method of claim 26, wherein determining is by detecting binding of the candidate MBL receptor antagonist to the MBL receptor and/or by detecting the inhibition of LCP associated complement activation.

28. The method of claim 26, wherein the MBL receptor is CK1 or a fragment of CK1.

29. A composition, comprising an isolated MBL receptor antagonist in a pharmaceutically acceptable carrier and a medicament for the treatment of an MBL mediated disorder.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Factor D
Lectin Pathway
MBL
Microbial Surfaces
MASP-1
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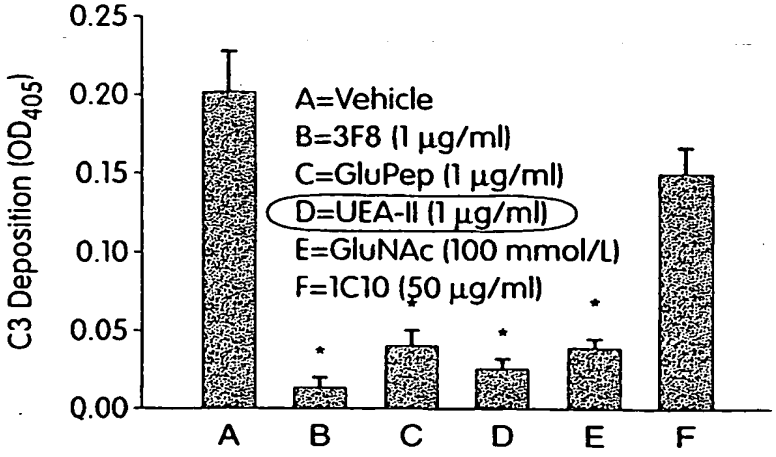
Fig. 1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0.10
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C=GluPep (1 µg/ml)
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E=GluNAc (100 mmol/L)
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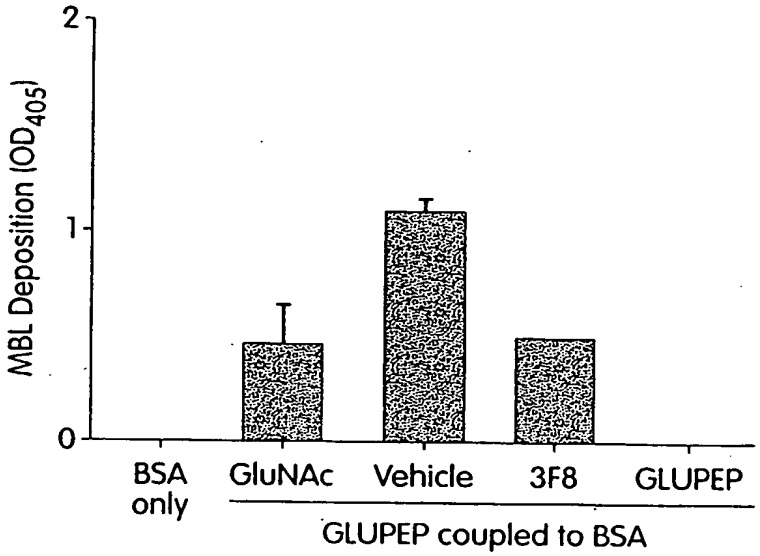
A
B
C
D
E
F

Fig. 2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Vehicle
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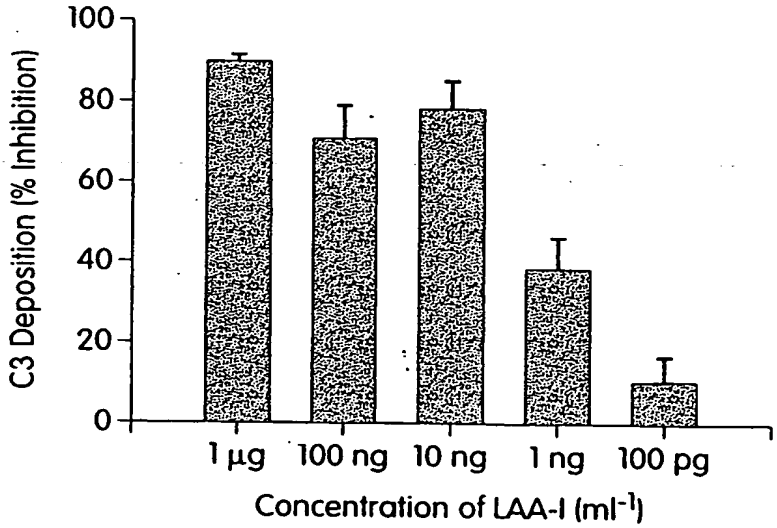
Fig. 3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Concentration of LAA-I (ml^{-1})

Fig. 4

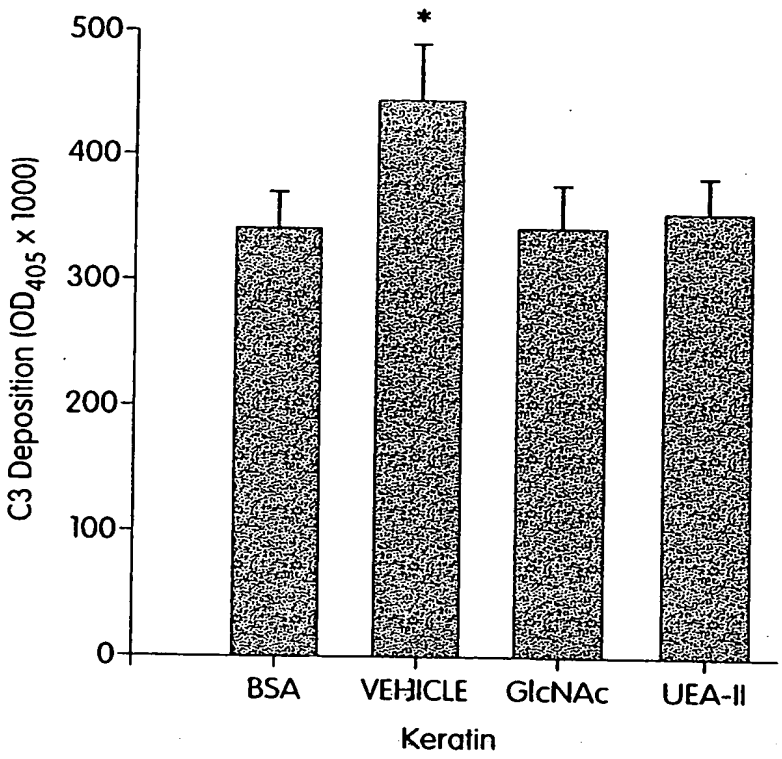
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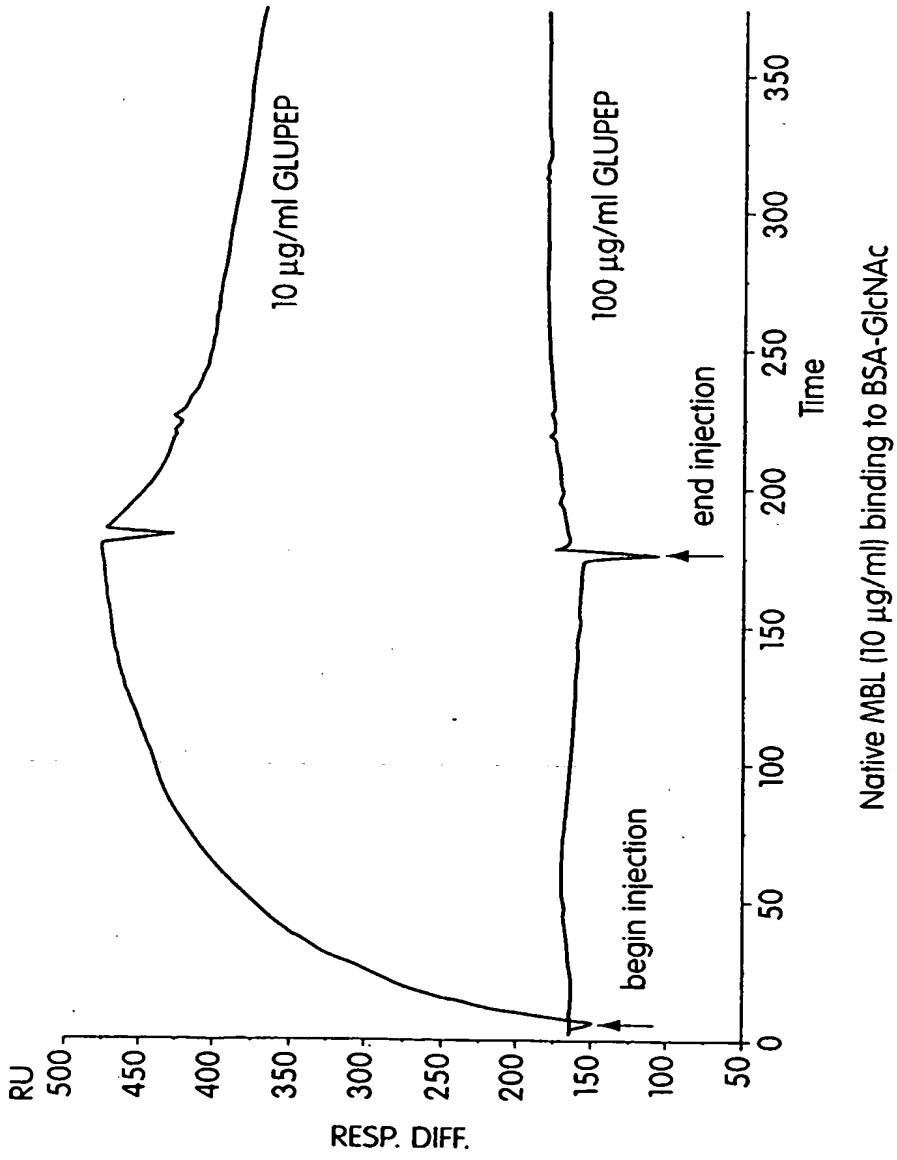
Fig. 5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100
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200
250
300
350
Time
10 µg/ml GLUPEP
100 µg/ml GLUPEP
begin injection
end injection
Native MBL (10 µg/ml) binding to BSA-GlcNAc

Fig. 6

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Lekowski, Robert

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INTERNATIONAL SEARCH REPORT

Intern Application No
PCT/US 00/22123

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/16 A61K39/395 A61P9/10 A61P11/00 A61P37/06
A61P7/02 A61P19/02 A61P9/00 A61P37/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, MEDLINE, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	COLLARD, C. D. (1) ET AL: "Endothelial reoxygenation activates the lectin complement pathway: Inhibition with anti-human mannose binding lectin (MBL) therapy." MOLECULAR IMMUNOLOGY, (MARCH APRIL, 1999) VOL. 36, NO. 4-5, PP. 278. MEETING INFO.: 7TH EUROPEAN MEETING ON COMPLEMENT IN HUMAN DISEASE HELSINKI, FINLAND JUNE 17-20, 1999 , XP000944464 the whole document --- -/--	1,8,9, 16-19, 25-27,29

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
12 December 2000	29/12/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	von Ballmoos, P

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	COLLARD, C. D. ET AL: "Reoxygenation of hypoxic human umbilical vein endothelial cells (HUVECs) activates the lectin complement pathway (LCP." FASEB JOURNAL, (MARCH 17, 1998) VOL. 12, NO. 4, PP. A29. MEETING INFO.: ANNUAL MEETING OF THE PROFESSIONAL RESEARCH SCIENTISTS ON EXPERIMENTAL BIOLOGY 98, PART 1 SAN FRANCISCO, CALIFORNIA, USA APRIL 18-22, 1998 FEDERATION OF AMERICAN SOCIETIES FOR EX, XP000944437 the whole document	1,8,9, 16-18, 25-27,29
X	WO 99 39209 A (HOLTZHAUER MARTIN ;BIOGENES GMBH (DE); OVODOV SERGEJ (DE); KNOLL A) 5 August 1999 (1999-08-05) abstract	20,21, 23,24
A	TURNER M W: "Mannose-binding lectin: the pluripotent molecule of the innate immune system." IMMUNOLOGY TODAY, (1996 NOV) 17 (11) 532-40. REF: 76 , XP000944318 cited in the application the whole document	1-29
A	LHOTTA K ET AL: "Glomerular deposition of mannose - binding lectin in human glomerulonephritis." NEPHROLOGY, DIALYSIS, TRANSPLANTATION, (1999 APR) 14 (4) 881-6. , XP000944395 the whole document	1-29
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INTERNATIONAL SEARCH REPORT

Intern I Application No
PCT/US 00/22123

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	LEKOWSKI, ROBERT W. (1) ET AL: "Ulex europaeus agglutinin II (UEA-II) is a novel, potent inhibitor of complement activation on human endothelial cells." CIRCULATION, (NOV. 2, 1999) VOL. 110, NO. 18 SUPPL., PP. I.259. MEETING INFO.: 72ND SCIENTIFIC SESSIONS OF THE AMERICAN HEART ASSOCIATION ATLANTA, GEORGIA, USA NOVEMBER 7-10, 1999 , XP000944434 the whole document ---	1,2, 6-12, 16-27,29
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INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern: d Application No
PCT/US 00/22123

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		EP 1053476 A	22-11-2000
WO 0035483 A	22-06-2000	NONE	